Root Flavonoids: their Transport and Role in Intra and Extra Cellular Signalling

Dissertation
zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

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Zürich, 2006

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ACKNOWLEDGMENTS

I would like to express my sincere gratitude to everyone who made this possible…

First and foremost, my thanks go to **Enrico** for his constant support and for truly understanding me in each different situation I experienced in the last 4 years, beyond the scientific subjects. My deepest appreciation for letting me think on my own, even when our ideas where not on the same track. On the other hand, thank you for dropping in the right tips at the right moments.

I wish to thank you the **NCCR** Plant Survival Doctoral School. Being a member of this school has provided me with invaluable opportunities to bolster my scientific knowledge and my technical skills, representing the first step towards a future career in academic science.

I am grateful to **Markus Geisler** and **Markus Klein** for fruitful collaborations, helpful and motivating discussions and for their constant help and support.

My sincerest thanks go to **Christoph Ringli** for fruitful collaboration and for having shared with me the discovery that flavonoids have a more than ever expected impact on plant growth and development.

I want to express my gratitude to the exceptional "white lupin team": **Laure** for making me realize that you can look at the world from very different points of view; and **Nicola** for being silently but constantly by my side and for the friendship we share.

Many thanks to my friends the "little hobbits" **Magali** and **Ulrike**, for their boundless energy and enthusiasm and for holding me in all the difficult situations I faced. Thank you for giving me the strength to go on.

My deepest appreciation goes to **Vincent**, my trusted friend, for its generous and precious technical assistance, and especially for making me feel at home during the many hours of work together.

My thanks also to **Mika Jasinski**, my friend and lab-mate, for all the technical tips, suggestions and support during the many unsucceful days in the lab.

Thanks to all present and former members of the **Hobbits's lab** and **Geisler's lab** for making my daily life in the lab easier and happier. In particular, I am grateful to **Aurelien**, the happy, fatty, lazy boy, very generous and always ready to help; and to **Sina** for sharing her smiling approach to life with me and for the help in translating the thesis summary. I will never learn German!

Thanks to all the **"Klein girls"**, and especially to **Krasi** because the last 4 years have seen us been asked to travel parallel and sometimes overlapping path, and we did it with great respect and friendship.

Many thanks to **Felix**, **Monika** and **Shaun** for their constant help in dealing with the fascinating and complex world of the HPLC.

Deep thanks to all the people in the **Institute** for the nice chats, discussions and help, and special thanks to the werkstatt staff for the amaizing work they do every day.

I am grateful to all the collaborators, especially to **Laurent** who made me understand how much interesting and constructive is communicating with scientists coming from different research fields.

Thanks **Nina** for the precious help in the formatting of my thesis. Whitout you, I would still be hanging around with all my word files…

My sincere thanks go to all the **friends** (Italian and not) that I met in Zurich for making the last years of my life full of joy.

A deep thank goes to **my family**. Although far from my daily life, they are always by my side. I love you.

Finally, my best gratitude is to **Massimo**, extraordinary man who has touched my life, and since then is always present.

SUMMARY

Flavonoids are one of the largest and most widespread group of plant secondary compounds. They are not fundamental to cell survival, but are necessary components for survival in the rapidilly changing environment in which plants grow, representing an essential part of the adaptation mechanisms. The diversity in these secondary metabolites is a result of simple differential modification of common precursors, leading to the formation of compounds having potentially divergent biological activitities, with specific and non-specific effects on intra and extra-organismal plant signalling mechanisms. From an evolutionary perspective it is clear that this strategy maximises the usage of secondary compounds with a minimal expenditure of energy. A whealt of information has been collected on the role of flavonoids in the biology of many different plant species. However, because of the large number of different flavonoid compounds that the plants can accumulate, many of their possible activities have not been discovered yet.

In the present work, we investigated several aspects of root flavonoid biology and biochemistry. In the first period of my PhD work, in collaboration with two other PhD students in the laboratory, I developed a project about the characterization and the structural elucidation of the major isoflavonoids in white lupin cluster roots and their potential role in the rhizosphere as antifungal compounds. We showed that genistein and hydroxygenistein, as well as their glycosylated conjugates, were the major flavonoids present in white lupin cluster roots. While internal contents remained stable during cluster root development, the amounts of isoflavonoids secreted varied in function of cluster roots stage. Highest secretion was observed at the juvenile and immature stages, preceding the peak of organic acid exudation occurring at the mature stage. Our results showed that micoorganisms in the rhizosphere were influenced by the secretion activity of cluster roots. In particular, in some fungal strains a stimulation of sporulation could be observed and *Fusarium* species seemed to be the most susceptible to white lupin's isoflavonoids. Fungal sporulation can be viewed as a stress response and with respect to the microbial degradation of secreted citrate, sporulation is certainly beneficial to the plant since spores represent a dormant stage in the life cycle of fungi. To our knowledge this is the first report of a role for isoflavonoids in protection of microbial organic acid degradations in cluster roots of white lupin.

How flavonoids and root exudates in general are secreted into the rhizosphere has been poorly investigated. In collaboration with Professor Vivanco from the Colorado State University, we compared by an HPLC-MS approach the root exudation profiles of seven ABC transporter mutants with those of the wild type, and found that three phytochemicals were missing in the root exudates of the various mutants. All tested mutants were deficient in either one or two compounds, and the same compound was sometimes missing in more than one mutant, indicating that more than one ABC transporter can be involved in the secretion of a given phytochemical or that a transporter can be involved in the secretion of more than one secondary compound. These results support the putative implication of different members of ABC tranporters in the secretion of phytochemicals from Arabidopsis roots.

In the second part of my PhD work, I investigated the role of flavonoids in plant development, especially in their regulation of polar auxin transport in plant cells, including the characterization of a new auxin transport protein, AtPGP4. We showed that *aux1* and *pin2*, two Arabidopsis polar auxin transport agravitropic mutants, have impaired patterns of flavonol glycosides. We found that exogenous flavonoids can partially rescue agravitropic phenotypes of *aux1* and *pin2* roots, by triggering the formation of lateral auxin gradients in a PIN2-independent manner. Our results suggest that flavonoid-dependent redundant auxin pathways are able to functionally substitute PIN2 action.

We provided several lines of evidences suggesting that AtPGP4, a new member of the PGP subfamily of the ABC transporter family in *Arabidopsis*, is involved in root development and in auxin transport processes as previously shown for AtPGP1. In contrast to PGP1, the reduced root IAA uptake capacities of the *pgp4* mutants compared to the wild type, as well as the fact that AtPGP4 confers hypersensitivity to the IAA sensitive *yap1-1* yeast mutant, suggests that PGP4 acts as an uptake protein. Our data demonstrated for the first time that an ABC transporter catalyzes the cellular import of auxin.

ZUSAMMENFASSUNG

Innerhalb der Klasse der pflanzlichen Sekundärstoffe bilden die Flavonoide die grösste und umfassenste Gruppe. Zwar sind sie für die Zelle nicht überlebenswichtig, dennoch sind sie ein wichtiger Bestandteil des pflanzlichen Anpassungsmechanismus, welche ein Überleben der Pflanze in den rasch ändernden Umweltbedingungen ermöglicht. Die sekundären Metaboliten verfügen über eine grosse Diversität, eine Folge von vielzähligen, einfachen Veränderungen eines gemeinsamen Vorläufers, wodurch sie in diverse biologische Vorgänge involviert sind. Diese können spezifische oder unspezifische Funktionen innerhalb des pflanzlichen Signalmechanismus übernehmen. Aus evolutionsbiologischer Sicht lässt sich schliessen, dass die Sekundär Metaboliten eine maximale Verwendung geniessen, welche durch einen minimalen Aufwand an Energie erlangt wurde. Nicht nur aus diesem Grund wurde in den letzten Jahren eine Fülle von Informationen zusammengetragen, welche die Funktion der Flavonoide in den verschieden Pflanzenspezies untersuchen. Trotz allem sind noch viele grundlegende Fragen über die diversen Funktionen der Flavonoide offen, da sich eine Vielzahl von verschieden Stoffen in hohen Konzentrationen in der Pflanze anreichern können.

In unserer derzeitigen Arbeit haben wir diverse Aspekte der flavonoiden Wurzelbiologie untersuchen können. In dem ersten Teil meiner Dissertation habe ich in Zusammenarbeit mit zwei weiteren Doktoranden unseres Labors die Hauptkriterien und die strukturellen Zusammenhänge der wesentlichen Isoflovonoide in der Clusterwurzel der Lupine und deren potentielle Rolle in der Rhizosphäre als antifungizider Stoff, erarbeiten können. Wir konnten zeigen, dass Genistein und Hydrogenistein, so wie auch dessen glycosyliertes Konjugat, die dominierenden Flavonoide in der Clusterwurzel der Lupine bilden. Obwohl die interne Konzentration während der Clusterwurzelbildung stabil bleibt, so variiert doch die sekretierte Menge an Isoflavonoiden je nach Entwicklungsstadium der Clusterwurzel. Die höchste Sekretionsrate konnte in den frühen und juvenilen Entwicklungsstadien beobachtet werden, welche einer Aussonderung an organischen Säuren in der ausgewachsenen Wurzel vorangeht. Ins besondere konnte in einigen Pilzen die Sporulation stimuliert werden. Auf Grund dieser Beobachtungen erschien uns *Fusarium* am empfänglichsten für die Isoflavonoide der weissen Lupine (*Lupinus albus*). Pilzsporulation kann als Antwort auf Stress erklärt werden vor allem in Bezug auf die mikrobielle Degradation von sekretiertem Zitrat. In diesem Sinne ist die Sporulation durchaus von Nutzen für die Pflanze, zumal die Sporen

die Ruhephase des Lebenszyklus eines Pilzes repräsentieren. Wir gehen davon aus, dass wir die ersten Daten liefern können, welche eine Schutzfunktion von Isoflavonoiden vor Degradierung durch mikrobielle organische Säuren in der Clusterwurzel von der weissen Lupine belegen.

Wie die Sekretion von Flavonoiden und anderen Wurzelabsonderungen in die Rhizosphäre im Detail von statten geht, wurde bis heute nur in einem geringen Umfang untersucht. In Zusammenarbeit mit Professor Vivanco von der Colorado State University, haben wir durch gezielte HPLC-MS Verfahren, Profile von Wurzelausscheidungen in Wildtyppflanzen und sieben verschieden ABC-Transpoter-Mutanten untersuchen und vergleichen können. Somit konnten wir die Abwesenheit von drei Phytochemikalien in den Wurzelausscheidungen der verschiedenen Mutanten zeigen. Alle von uns getesteten Mutanten zeigten einen Mangel in entweder einem oder zwei Stoffen, welcher wiederum in einer oder mehreren Mutanten fehlte. Dies könnte bedeuten, dass mehr als nur ein ABC-Transporter an der Ausscheidung von einer bestimmten Phytochemikalie beteiligt oder das ein Transporter an der Sekretion mehrerer Sekundär Stoffe involviert ist. Diese Ergebnisse unterstützen die Annahme, dass mehr als nur ein ABC-Transporter an der Sekretion von Phytochemikalien in der Wurzel von *Arabidopsis* beteiligt ist.

In dem zweiten Teil meiner Doktorarbeit, habe ich die Rolle der Flavonoide während der Pflanzenentwicklung untersucht. Meine besondere Aufmerksamkeit habe ich der Regulierung des polaren Auxin Transports in Pflanzenzellen gewidmet, welches die Charakterisierung des neuen AtPGP4 Auxin Transportproteins beinhaltete. Wir konnten zeigen, dass *aux1* und *pin2,* zwei agravitrope Arabidopsis Mutanten des polaren Auxin Transports, eine beeinträchtigte Verteilung von flavonolen Glycosiden zeigen. Desweiteren konnten wir zeigen, dass eine Behandlung mit exogenen Flavonoiden den agravitropen Phänotyp der *aux1* und *pin2* Wurzeln wieder herstellen kann, wodurch ein lateraler Auxin-gradient unabhängig von PIN2 gebildet wird. Unsere Ergebnisse lassen vermuten, dass Flavonoid abhängige, redundante Auxinwege die Funktion von PIN2 ersetzten können.

Wir lieferten mehrere Beweise die annehmen lassen, dass AtPGP4, ein neues Mitglied der PGP-Subfamilie der ABC-Transporter in Arabidopsis, eine Rolle während der

Wurzelentwicklung und des Auxin Transports spielt, was schon vorher für AtGP1 gezeigt werden konnte. Die reduzierte Aufnahme von IAA der *pgp4* Mutanten im Vergleich zum Wildtyp und die Hyperssensibilität von AtPGP4 gegenüber der IAA sensitiven *yap1-1* Hefemutante, lässt annehmen, dass PGP4 im Gegensatz zu PGP1 ein Aufnahmeprotein ist. Unsere Daten zeigen zum ersten Mal, dass ein ABC-Transporter die Aufnahme von Auxin in die Zelle katalysiert.

1 INTRODUCTION

Across evolution, plants have developed a complex network of metabolic pathways, mainly classified in primary and secondary metabolism. Primary metabolism refers to the anabolic and catabolic processes required for respiration, nutrient assimilation, and growth/development, namely those processes required for cell maintenance and proliferation. In contrast, secondary metabolism refers to compounds present in specialized cells that are not necessary for cell survival but are thought to be required for the plant survival in the environment (Kossel, 1891). A primary difference between plants and animals is that plants are in most cases static, unable to move and literally rooted to the spot. A consequence of this life style is that plants are unable to avoid changes in their environment; hence, they always need to accommodate them. One of the major adaptive responses of plants to environmental changes is the activation of secondary metabolic pathways to synthesize protective compounds.

In this respect, secondary metabolites are an essential part of plant success in adapting to life as sedentary organisms living in diverse and inconstant surrounding. They are believed to aid plant fitness and survival by preventing insect herbivory and pathogen attack as well as support reproduction through providing pollinator attraction as either floral scent or colour. The requirement for these metabolites to have highly diverse biological activities has led the plant to accumulate a vast catalogue of secondary compounds. The number of secondary metabolites in vascular plants is at least several hundred thousand (Dixon and Strack, 2003; Wink, 1988).

Most secondary plant products can be classified into three maior groups: alkaloids, isoprenoids (terpenes) and phenolic compounds (mostly phenylpropanoids). They vary in structure from simple aliphatic molecules to complex polycyclic aromatic compounds. However, most of their structural diversity is generated by differentially modifying common backbone structures of precursors, with the derived compounds having potentially divergent biological activities. One explanation for this modular diversity is that selection favours plants with newly derived defences when insects or other pests have evolved the ability to overcome existing defences (Ehrlich and Raven, 1964). New defensive compounds can be synthesized by structurally modifiying a toxic compound to evade the pest's counter-defence while maintaining the compounds toxic activity. The reiteration of this process over millennia may explain the vast range of plant secondary metabolic chemistry.

This introduction is organized in four sections. The first part (**section 1.1**) is dedicated to a survey of the state-of-the-art of flavonoids research, with major emphasis on work done on the model plant *Arabidopsis thaliana*. The following sections are devoted to the description of the biological activities of flavonoids as signal molecules: while **section 1.2** focuses on plant-microorganism interactions, referring to white lupin rhizosphere as a paradigm, **section 1.4** describes the role of flavonoids as regulators of polar auxin transport in plant development and during root gravitropism. Within the latter section (namely, **1.4.6**), I will briefly report on how flavonoids can influence cell growth and development through auxin-induced and auxin-indipendent processes. A special section (**section 1.3**) discusses the involvement of two classes of membrane transporters, the ABC (ATP binding cassette) and the MATE (Multidrug And Toxic compound Extrusion) transporters, in the exudation of root phytochemicals.

1.1 FLAVONOIDS

Flavonoids have been recognized as one of the largest and most widespread group of secondary metabolites. Since the first identification in the mid-1800s, about 9000 flavonoids have been identified in plants (Whiting, 2001). The biological role of flavonoids has attracted much attention to these secondary compounds over the years. From the first description of acid and base effect on plant pigments by Robert Boyle in 1664 to the characterization of structural and regulatory genes in the late 20th century, a wealth of information has been collected on the structures, chemical activities, and biosynthesis of these compounds. In the next paragraphs I will survey the state-of-the-art of flavonoids research, with emphasis on work of the last decade and on *Arabidopsis thaliana* as a model system.

1.1.1 Chemical structure and classification

Flavonoids consist of several classes of structurally similar compounds having a common C_6-C_3-C_6 flavone skeleton (Figure 1) in which the three-carbon bridge between the phenyl groups is commonly cyclized with oxygen. The major classes are flavones, isoflavones, flavonols, anthocyanins, flavanones, catechins, chalcones, and aurones, and are differentiated according to the degree of insaturation and degree of oxidation of the three-carbon segment (Hughes et al., 2001; March et al., 2004). The basic structures are

reported in Fig. 1. Within the various classes, further differentiation is possible based on

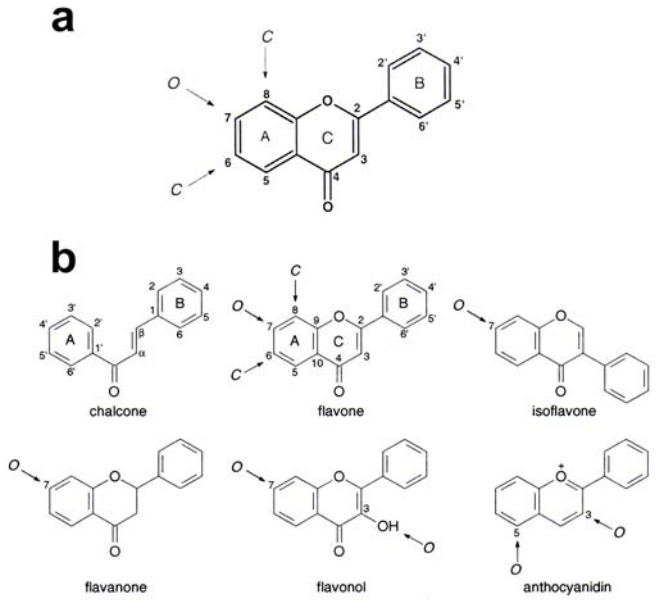


Figure 1. (a) General structure of flavonoids, the C15 (C6-C3-C6) base skeleton. Arrows indicate the main glycosylaton sites. From Cavaliere *et al.*, 2005. **(b)** Basic structures of the main classes of flavonoids. Common O- and C-glycosylation positions are indicated by an arrow. From Cuyckens and Claeys, 2004.

the number and nature of substituent groups attached to the rings (Robards and Antolovich, 1997). Flavonoids can exist both as free aglycones and as glycosidic conjugates (He, 2000; Stobiecki et al., 1999); moreover, various other modified forms (hydroxylated, methylated, prenylated) may occur (Cuyckens and Claeys, 2004; Iwashina, 2000). The effect of glycosylation is to render the flavonoid less reactive, more polar and soluble, so that this modification can be regarderd as an essential step of protection in plants to prevent cytoplasmatic damage and to store the flavonoids safely in the cell vacuole (Cuyckens and Claeys, 2004; Justesen et al., 1998). Therefore, in plants flavonoids occur almost exclusively as glycosidic conjugates.

In flavonoids *O*-glycosides, one or more of the aglycone hydroxyl groups is bound to a sugar with formation of an O-C acid-labile hemiacetal bond. In principle, any of the hydroxyl groups can be glycosylated, but certain positions are favored and specific for each subfamily: e.g., 7-hydroxyl group in flavones, flavanones and isoflavones, the 3- and 7-hydroxyls in flavonols and flavanols, and the 3- and 5-hydroxyls in anthocyanidins are common glycosylation sites (Cuyckens and Claeys, 2004; Iwashina, 2000; Robards and Antolovich, 1997). Glycosylation may also take place by direct linkage of the sugar to the basic nucleus of the flavonoid via an acid-resistant C-C bond, to form flavonoid *C*-glycosides. To date, *C*-glycosylation has only been found at the C-6 and/or C-8 positions of the flavonoid nucleus. Glucose is the most common encountered sugar, followed by

galactose, rhamnose, xylose and arabinose, whereas glucuronic and galacturonic acids are rare.

Flavonoids isolation and structure elucidation

Isolation and structure elucidation of flavonoids are the initial steps to understanding their significance and action. For the investigation of structure-activity relationships, a sophisticated high-resolution technique is required because of the number and diversity of flavonoids. Modern mass spectrometric techniques, particularly in coupled modes with HPLC or GC, are very well suited for the analysis of flavonoids in plants, since they can provide significant structural information on small quantities of pure samples as well as on mixtures. HPLC combines the advantages of simultaneous separation and quantification without the need of preliminary derivatization. Reversed-phase chromatography (RPC) has invariably been the method of choice for the separation of flavonoids, usually on C_8 or C_{18} columns used in conjunction with aqueous mobile phases and methanol or acetonitrile as organic modifiers (Pietta et al., 1995). Small amounts of acetic acid, formic acid of phosphate buffers incorporated in the mobile phase improve separations markedly (Lamuelaraventos and Waterhouse, 1994).

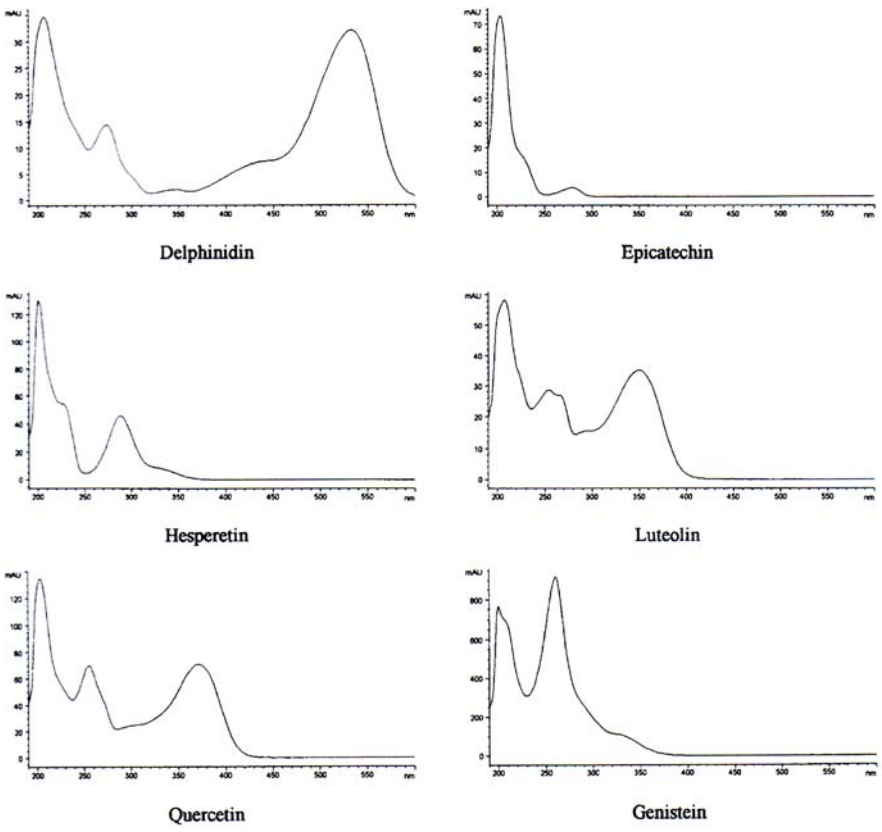


Figure 2. UV-vis spectra of the anthocyanidin delphinidin, the catechin epicatechin, the flavanone hesperetin, the flavone luteolin, the flavonol quercetin, and the isoflavone genistein. From Merken and Maryland, 2000.

Under the usual reversed-phase conditions, the more polar compounds are generally eluted first (Wulf and Nagel, 1976). Thus, diglycosides precede monoglycosides, which precede aglycones. The elution pattern for flavonoids containing equivalent substitution patterns is flavanone followed by flavonol and flavone. This elution pattern holds for both aglycones and glycosides (Park et al., 1983).

Detection of the eluted species has been based on measurement of absorption at characteristic wavelengths (Gao and Mazza, 1994). Phenols absorb in the ultraviolet (UV) region. Two absorption bands are characteristic for flavonoids. Band II, with a maximum in the 240-285 nm range, is believed to arise from the A-ring. Band I, with maximum in the 300-550 nm range, presumably arises from the B-ring. The precise positions and relative intensities of these bands provide valuable information on the nature of the flavonoid and its oxygenation pattern (Merken and Beecher, 2000). Figure 2 shows UV-vis spectra representative of each of the major subclasses of monomeric flavonoid aglycons. In table 1 the UV absorption maxima of each compound class are reported.

Table 1. UV absorption maxima of various classes of phenolic compounds. From Robards and Antolovich, 1997

	Absorption maxima/nm	
Compound class*	Band II	Band I
Simple phenols	266–295	
Phenolic acids	235–305	
Hydroxycinnamic acids	227–245, 310–332	
Hydroxycoumarins	*ca.* 210, 250–260, 280–303	312–351
Flavonoids—		
Flavones, biflavones	250–280	310–350
Isoflavones	245–275	310–330
Flavonols	250–280	350–385
Flavanones	275–295	310–330
Chalcones	240–260	365–390
Aurones	240–270	390–430
Anthocyanins	265–275	465–560

* Usual solvent is methanol with the exception of methanolic HCl for anthocyanins.

After HPLC, flavonoid extracts can be further purified for mass spectrometry (MS). The mass spectrometer is a universal detector that can achieve very high sensitivity and provide information on the molecular mass and on structural features. More detailed information can subsequently be obtained by resorting to tandem mass spectrometry (MS/MS) in combination with atmospheric pressure ionization (API) sources. MS/MS analysis can be pursued for further structure characterization. Additionally, it can be used to determine the occurrence of previously identified compounds. The highest sensitivity is obtained using as API source the electrospray ionization (ESI) in the negative ion mode (Rauha et al., 2001). The negative ion mode results in limited fragmentation, making it most suited to infer the molecular mass of separated flavonoids, especially in case where concentrations are low. In a structure characterization of flavonoids, the information that

can be obtained is: (1) the aglycone moiety, (2) the types of carbohydrates or other substituents present, (3) interglycosidic linkage and (4) attachment point of the substituents to the aglycone.

Part of my PhD work focused on the isolation, quantification and identification by HPLC-(-)-ESI-MS of major flavonoids structures in two plant model systems: *Arabidopsis thaliana* (section 4.6) and white lupin, *Lupinus albus L.* (section 4.1). In the next two paragraphs, I therefore would like to introduce the reader to the major flavonoids so far identified in these two species.

Arabidopsis flavonoids

The major flavonoids compounds in *Arabidopsis thaliana* are the flavonols (D'Auria and Gershenzon, 2005; Rohde et al., 2004; Veit and Pauli, 1999). The most common flavonols are three kaempferol glycosides (mainly glucose and rahmnose), but quercetin glycosides can also accumulate. The identities of the metabolites suggest that rhamnose may be the preferred sugar attached to the 7 position of the aglycone and that further glucose or rhamnose residues are then added to form di- and tri-glycosides (Graham, 1998). Although found predominantly in shoots, flavonol glycosides can also be induced in the roots after exposure to light (Hemm et al., 2004) and have also been found as root exudates (Narasimhan et al., 2003). The flavonol aglycones kaempferol and quercetin are also present in *A. thaliana*, especially in early developmental stages (Peer et al., 2001), as is the methylated flavonol isorhamnetin (Muzac et al., 2000). Another group of flavonoids that are present in *Arabidopsis* are the anthocyanins, the major red, purple and blue pigments of plants. The anthocyanins are produced in variable amounts in leaves and stem depending on light level and nutritional conditions, and most of them are cyanidin derivatives (Tohge et al., 2005). The major anthocyanin in *Arabidopsis* has a cyanidin core with four attached sugars and a single *p*-coumaroyl, sinapoyl, or malonyl residue (Bloor and Abrahams, 2002). The seeds contain a group of polymeric flavonoids, the proanthocyanidins (PAs) or condensed tannins. These are formed from epicatechin-type monomers (± 8 monomers per molecules, (Abrahams et al., 2003)). PAs are colourless flavonoids and accumulate in specific cells of the inner tegument (mainly endothelium) and the calaza (Debeaujon et al., 2003). Very recently, a comprehensive characterization of more than 26 different flavonoids that accumulate during seed development and maturation of *Arabidopsis thaliana* has been published (Routaboul et al., 2006).

Interestingly, a novel group of four biflavonols (dimers of quercetin-rhamnoside) was detected. Moreover, about 85% of the flavonol glycosides from seed are derived from quercetin, quercetin-3-*O*-rhamnoside being the major flavonol specie, which is in contrast to *Arabidopsis* leaves, stems and flowers in which most flavonol glycosides are derived from kaempferol (Peer et al., 2001; Pelletier et al., 1999; Veit and Pauli, 1999).
Large-scale metabolic profiling approach that combines separation by capillary liquid chromatography with the high resolution, high sensitivity, and high mass accuracy of quadrupole time-of-glifht mass spectrometry has been apply to *Arabidopsis* roots and leaves exctracts, allowing the identification of about 2000 different mass signals (von Roepenack-Lahaye et al., 2004). This work is a good example of how metabolome analysis provides innovative and powerful means of identifying new plant metabolites.

White lupin isoflavonoids

Plants from the genus *Lupinus* accumulate a broad range of isoflavonoids, mainly isoflavones such as genistein and 2'-hydroxygenistein. These two compounds maybe further modified by isopentenyl (prenyl) groups at different positions on the skeleton, giving rise for example to wighteone and luteone in white lupin (*Lupinus albus*) (Ingham et al., 1983; Tahara et al., 1989). In health mature lupin plants, most isoflavonoids are accumulated as glucosides in the vacuole (Wojtaszek et al., 1993), and genistein and 2'-hydroxygenistein could serve as substrates for the synthesis of prenylated derivatives following their release from respective glucosidic conjugates. LC combined with UV detection was used to study isoflavonoid profiles in roots of white and yellow lupin seedlings elicited with biotic or abiotic elicitors, known for their stimulating activity on isoflavonoids accumulation (Gagnon and Ibrahim, 1997). In this study the main interest was towards analyses of free aglycones present in the plant tissue. LC/ESI/MS systems were used for the detection of isoflavonoid glycosides and free aglycones in extracts from white lupin plants (Bednarek et al., 2001; Stobiecki et al., 1999). Profiles of the flavonoid glycosides differ in various organs of lupin seedlings. The highest total amount of flavonoid glycosides, expressed as genistein 7-*O*-glucosides equivalents, is found in roots and leaves, lower amount are found in stems. Glycosides of kaempferol and isorhamnetin are present only in the green parts, while apigenin 7-*O*-glucoside was detected only in the roots. Relative quantities of acylated isoflavonoid glucosides vary in different organs. The highest ratio of 6"-*O*-malonylated di- and monoglucosides of

isoflavonoids to non acylated compound is found in roots and stem; only in leaves the amounts of non-acylated glycosides exceed those of malonylated compounds. Free aglycones constitute at maximum few percent of the total amount of isoflavonoids; the highest ratio of free aglycones to glycosides is found in the roots (Wojtaszek and Stobiecki, 1997). HPLC analysis combined with a photodiode array (PDA) detection system was applied to quantify the changes in isoflavonoids constitutents in white lupin tissues during development (Katagiri et al., 2000). This analysis focused on young legumes (pods and seeds) during maturation, and soaked, germinating seeds. In developing legumes, genistein and 2'-hydroxygenistein, as well as their prenylated derivates, together with minor amounts of glucosides, are present in pods as major component. Only minute amounts of isoflavonoids are detectable in ripening seeds. When soaked with water, mature lupin seeds start rapidally to synthesize simple isoflavones and accumulate large amounts of genistein-7-*O*-glucoside and its 6"-*O*-malonyl derivative.

1.1.2 Flavonoid metabolism

Flavonoid biosynthesis is complex and numerous enzymatic steps are involved. Flavonoid synthesis is organ- and tissue-dependent, and it is affected by environmental conditions, such as light intensity, temperature and nitrogen nutrition (Woo et al., 2005). Thus, information on flavonoid biosynthesis is essential to understand the interaction between plants and their environment.

In the early steps of flavonoids biosynthesis, phenylalanine derived from the shikimic acid pathway is converted to coumaroyl-CoA by phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase, and 4-coumarate:CoA ligase. Chalcone synthase (CHS), the first committed enzyme for flavonoid synthesis, results in the condensation of coumaroyl-CoA with three molecules of malonyl-CoA to form naringenin chalcone. Naringen chalcone is then converted to naringenin by chalcone isomerase. Naringenin is further converted by glycosylation, acylation, and methylation of its three rings. The end result of all these enzymatic steps is the synthesis of substituted flavones, flavonols, catechins, deoxyflavonoids, and anthocyanins (Figure 3) (Springob et al., 2003; Winkel-Shirley, 2002). Isoflavonoids are formed from naringenin or deoxyflavonoids by an aryl migration of the B-ring to the 3-position, followed by hydroxylation at the 2-position,

which is catalyze by isoflavone synthase (IFS), a P450 enzyme. The 2-hydroxyisoflavone is unstable and is dehydrated to yield isoflavone (Dixon and Ferreira, 2002).

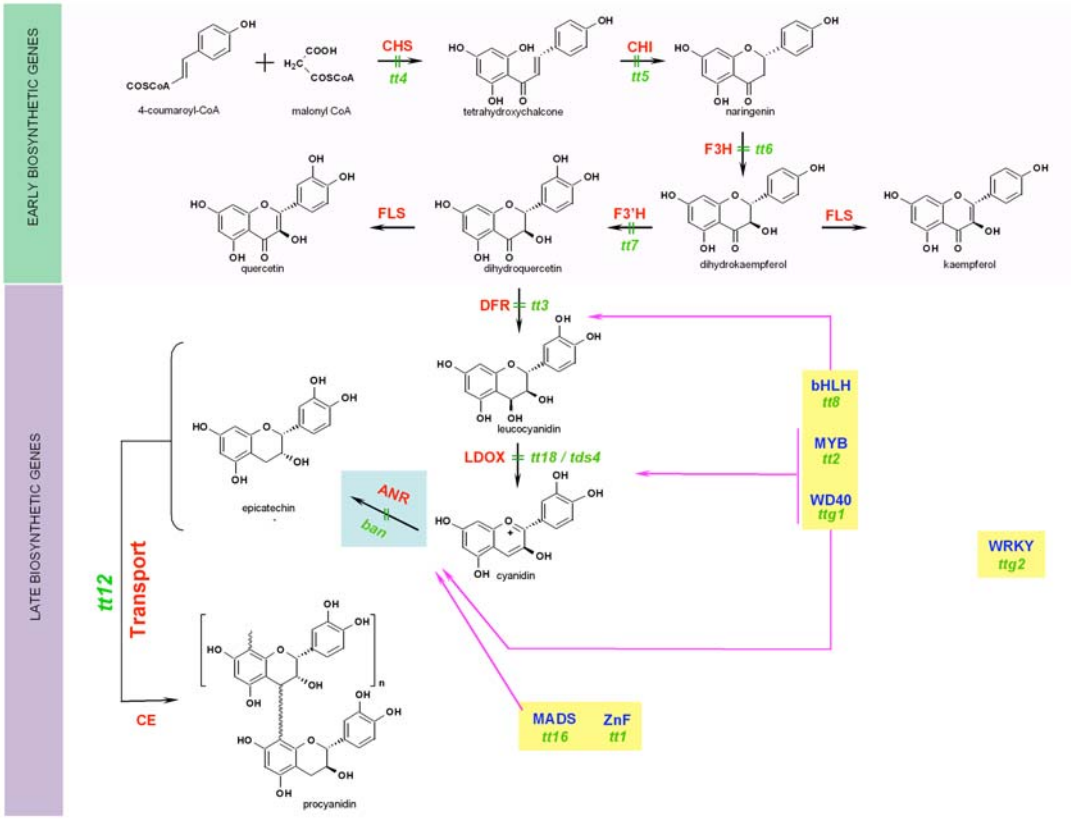


Figure 3. Simplified scheme of flavonoid biosynthesis and its regulation in *Arabidopsis*. From Marinova and Debeaujon, unpublished.

In recent years, much effort has been directed at elucidating the flavonoid biosynthetic pathway from a molecular genetic point of view. Mutants affecting flavonoid synthesis have been isolated in a variety of plant species based on alteration in flower and seed pigmentation. *Arabidopsis* deletion mutants have greatly facilitated the analysis of the regulation and subcellular organization of the flavonoid pathway (Winkel-Shirley, 2001). One unique aspect of using Arabidopsis for studying flavonoid biosynthesis is that all but one (flavonol synthase, FLS) of the enymes of central flavonoid metabolism (leading to flavonols and anthocynins) are encoded by single-copy genes (Pelletier et al., 1999; Wisman et al., 1998), allowing null alleles that disrupt flavonoid biosynthesis throughout the plant to be isolated. Genetic loci for both structural and regulatory genes are scattered across the *Arabidopsis* genome and have been identified largely on the basis of mutations that abolish or reduce pigmentation in the seed coat. As a result, the loci were named *transparent testa* by Maarten Koornneef (Wageningen Agricultural University, The

Netherlands), who isolated many of the first mutants in this class in the '80s. An initial identification of 11 mutants has now been expanded to more than 24 members (*tt1-19* plus *fls1*, *ban*, *ttg1*, *ttg2*, *aha10*). 17 genes have been identified at the molecular level, among which 8 are structural genes (*CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, *LDOX*, *FLS* and *ANR*), 6 encodes regulatory proteins (TT1, TT2, TT8, TT16, TTG1, TTG2), and three are involved in flavonoid compartimentation (TT12, TT19 and AHA10).

The enzymes involved in transferring sugars from a range of nucleoside donors onto acceptor molecules, the glycosyltransferases (GTs), have been also intensively studied (Jones and Vogt, 2001; Li et al., 2001; Paquette et al., 2003), although the synthesis of several hundred different conjugates forms of flavonoids is still in its infancy. Family 1 of GTs (UGTs) utilizes small molecular weight compounds as acceptor substrates and UDP-sugars as donors, catalyzing the modifications of the precursors aglycone flavonoids. Several flavonoid GTs, primarily acting on anthocyanidin and anthocyanin acceptor substrates, have been characterize at the molecular level in a range of species including *Petunia hybrida* (Yamazaki et al., 2003) and *Vitis vinifiera* (Ford et al., 1998). In *Arabidopsis*, 4 different flavonoid glycosyltransferases, acting either on flavonols or anthocyanins have been identified and charactherize so far (Jones et al., 2003; Tohge et al., 2005).

The flavonoid biosynthetic machinery has been generally found in the cytoplasm, where it appears to be organized as a multienzyme complex both at the endoplasmatic reticulum (ER) and in as-yet-uncharacterized electron-dense particles (Winkel, 2004). The concept of multienzyme complex was first proposed by Stafford to explain the direct transfer, or channelling, of highly reactive and potentially toxic flavonoids intermediates between active sites without diffusion into the cytoplasm, and to substantiate a mechanism for coordinating the reactions of different metabolic pathways (Stafford, 1974). In the following years, a collection of experimental evidences (Burbulis and Winkel-Shirley, 1999; Saslowsky and Winkel-Shirley, 2001) has led to a model in which the flavonoid biosynthetic pathways are organized as a linear array of enzymes loosely associated with the ER and anchored to the membrane via the cytochrome P450-dependent monooxygenases, cinnamate-4-hydroxylase (C4H) and F3'H. However, it has been recently reported that at least two of the flavonoid biosynthetic enzymes (CHS and CHI) are located not only in the cytoplasm but also in the nucleus of several cell types in *Arabidopsis* (Saslowsky et al., 2005). Since a number of fluorescence microscopy studies have provided evidence for the accumulation of flavonoids in nuclei of diverse plants

species, including *Arabidopsis* (Buer and Muday, 2004; Hutzler et al., 1998; Kuras et al., 1999; Peer et al., 2001), it has been hypothesized that flavonoids located in the nucleus might be synthetized *in situ*, suggesting that differential targeting of the biosynthetic machinery may be used to regulate the deposition of plant secondary products at diverse sites of action whitin the cell.

Generally, flavonoid end products are thought to be transported from the site of synthesis in the cytoplasm to the final cellular destinations. In some tissues, such as the epidermis of leaves and flowers and endothelium of developing seed coat, flavonoids are transported primarily to the vacuole by processes that appear to involve a glutathione S-transferase (GST), and a glutathione pump belonging to the ATP-binding cassette (ABC) family of transporters or a toxic compound extrusion protein of MATE secondary transportes (Alfenito et al., 1998; Debeaujon et al., 2001; Marrs et al., 1995). In other tissues, a significant proportion of flavonoids is deposited in the cell wall (Markham et al., 2001) or secreted (Kuras et al., 1999). Vesicles apparently involved in transport of flavonoids to the cell periphery have been described in shorgum plants responding to fungal infection (Snyder and Nicholson, 1990) and maize cells induced to accumulate anthocyanins (Grotewold et al., 1998). This localization might occur via alternative secretory pathways not involving the Golgi (Lin et al., 2003). It has been proposed that a large proportion of flavonoids can even remain in the cytoplasm in some tissues (Hutzler et al., 1998).

The fluorescent dye diphenilyboric acid 2-aminoethyl ester (DPBA) provides a method to observe flavonoid accumulation in plant tissues *in vivo*. The fluorescence of flavonoids conjugated to DPBA (Sheahan and Rechnitz, 1992). Murphy et al. demonstrated tissue-specific localization of flavonoids in 4dag *Arabidopsis* seedlings (Murphy et al., 2000). Quercetin, kaempferol, and naringenin chalcone (NC) were shown to be concentrated in three tissues: the upper hypocotyl (UH), the hypocotyl-root transition zone (TZ) and the distal elongation zone. In a successive study from the same laboratory (Peer et al., 2001), aglycone flavonols have been shown to accumulate at the hypocotyl-root transition zone in a developmental and tissue-specific manner with kaempferol (yellow-green) in the epidermis, as a single ring of cells, and quercetin (gold) in the cortex, as a cone beneath the kaempferol containing ring, with an overall peak of fluorescence at 5dag. Kaempferol accumulates also in the root cap, while quercetin fluorescence was observed throughout the length of the root, with a pronounced accumulation in the root elongation zone. *tt* mutants flavonoid accumulation pattern has been also describe. Flavonoids accumulate

in the same regions in the *tt* mutants and in the wild type, suggesting that synthesis and end product accumulation occur in the same cells. However, in the stained *tt4* seedlings only autofluorescence of the chlorophyll and sinapate esters was observed, *tt3* accumulates a greater amounts of kaempferol and quercetin than in the wt and *tt7* accumulates kaempferol in the ring zone and cone zone of shoot-root transition zone.

1.1.3 Multiple biological roles of flavonoids

Although the most visible function of the flavonoids is the formation of the red and purple anthocyanin pigments, non visible flavonoid compounds also play central roles in the biology of plants. Nearly every class of flavonoids has been shown to have a biological activity, with specific and non-specific effects on intra- and extra-organismal plant signalling mechanisms. The role of flavonoids in extra-organismal plant signalling has been described extensively, but their role in intra- and intercellular signalling is not as well documented, although of increasing interest.

Flavonoid signalling via root exudates has been well studied in cases of pathogen defense, allelopathy and nodulation. In symbiotic N_2 fixation, the secretion of flavonoids and isoflavonoids by host plants is proposed to be among the earliest steps towards establishing the symbiotic relationship (Hirsch, 2004). Flavonoids activate rhizobial nodulation (*nod*) gene expression by interacting with the product of *nodD*, a bi-functional transcriptional repressor/activator of the *nod* genes. In response, the bacterial symbiont produces Nod factor, an oligosaccharide that elicits the early stages of nodule formation. The structural specificity of flavonoids is the determining factor for the interaction with the NodD regulatory protein in different *Rhizobium* species. Flavonoids have also been proposed to function as external chemical signal for mycorrhizal colonization (Harrison, 1999). Transcripts of enzymes encoding the flavonoid biosynthetic pathway, phenylalanine lyase (PAL), and chalcone synthase (CHS), but not the defense-specific enzyme isoflavone reductase, are induced specifically in cells containing arbuscules in *M. truncatula*. This induction may reflect biosynthesis of flavonoid compounds that stimulate the growth of mycorrhizal fungi rather than production of antimicrobial phytoalexins (Harrison, 2005). In addition, the root and shoot flavonoid composition is altered between colonized and non colonized plants, suggesting that flavonoids may mediate the colonization (Ponce et al., 2004). Under low phosphate conditions melons synthetize a *C*-glycosylflavone, isovotexin 2"-*O*-β-glucoside, which increase mycorrhizal colonization

(Akiyama et al., 2002). Flavonoids are also important in disease defense mechanisms. Ingress of phatogenic microbes is some plants can be curtailed in part by synthesis of flavonoid and isoflavonoid phytoalexins (Dixon and Paiva, 1995). Phytoalexins are produced after pathogen infection as a defense mechanism (Hammerschmidt, 1999). Moreover, flavonoids are among many of the allelopathic agents that plants produce to reduce competion. Flavones from rice leaves inhibited weed growth, but not rice biomass production (Kong et al., 2004); (-)-Catechin, kaempferol and dihydroquercetin in root exudates from the invasive species *Centaurea maculosa*, can trigger a wave of reactive oxygen species (ROS) and subsequent Ca^{2+} signalling leading to root death in sensitive plant species (Bais et al., 2003).

Flavonoids may act as well as internal physiological regulators or chemical messengers within the intact plant. One obvious function of flavonoids is as filter for UV radiation which requires a relatively large concentration of these compounds in plant epidermal cells (Li et al., 1993). Flavonoids contribute to a general reduction of reactive oxygen species (ROS), therefore protecting DNA and proteins damage, degradation of the photosystem II reaction center and membrane integrity. However, although ROS-mediated signal transduction mechanisms may be altered in a flavonoid-deficient background, flavonoids might be better regarded as part of the cellular context in which the response takes place as a specific signalling mechanism (Peer and Murphy, 2006). Flavonoids also accumulate in the anthers and pistil, and are involved in pollen development and germination (Mo et al., 1992). A flavonoid deficient mutant of petunia is male-sterile because its pollen fails to germinate (van der Meer et al., 1992). Exogenous application of kaempferol induces pollen-specific gene expression and restores synchronous germination and pollen tube growth (Guyon et al., 2000). However, this is not true for *Arabidopsis*, where the lack of flavonoids in flowers and stamens does not affect male fertility (Burbulis et al., 1996). Naturally occurring flavonoids have been proposed to be endogenous regulators of auxin transport in plants, because they are able to inhibit auxin efflux in tissue fragments and to displace the efflux inhibitor NPA (1-*N*-naphthylphthalamic acid) from its binding protein in membrane fractions (Jacobs and Rubery, 1988). Later, several others studies focused on flavonoids and auxin transport, showing that various flavonoids, mainly those belonging to the flavonols subfamily, can negatively regulate polar auxin transport and that environmental stimuli influence flavonoid accumulation, which in turn regulates auxin transport and relative physiological responses (Brown et al., 2001; Buer and Muday, 2004; Murphy et al., 2000).

1.2 ROLE OF FLAVONOIDS AS SIGNAL MOLECULES IN PLANT-MICROORGANISM INTERACTIONS: WHITE LUPIN AS A PARADIGM

Some of the most complex chemical, physical and biological interactions experienced by terrestrial plants are those that occur between roots and their surrounding environment of soil. The relathionship between plants and microorganisms is intense, complex and influenced by many different factors. In this section I will describe the plant-microbe interactions in the rhizosphere, and discuss the role of flavonoids as extracellular signaling molecules. I will in particular focus on white lupin's rhizosphere as a model system.

1.2.1 Potential plant-microbe interactions in the rhizosphere

From the root surface up to about 2 mm away from the root surface, a zone of soil is located that is significantly influenced by living roots and is referred to as the "rhizosphere" (Uren, 2000). The soil rhizosphere is important in terms of root growth, exudate production and community development of macro and micro biota. The chemical and biological characteristics of this zone differ drastically from those of bulk soil. For example, the pH of the rizospheric soil can be much lower than of the bulk soil (Hubel and Beck, 1993). The nutrient status of this region is also affected by the presence of roots, since roots remove dissolved nutrients from the soil solution and also solubilize nutrients from soil minerals.

The rhizosphere is densely populated and roots must compete with the invading root systems of neighboring plant species for space, water, and mineral nutrients, and with soil-borne microorgasnisms on an abundant source of organic material (Ryan et al., 2001a). Thus, root-root and root-microbe communications are likely continuos occurrences in this biologically active soil zone, and they can either be positive (symbiotic) to the plant, such as the association of epiphytes, mycorrhizal fungi, and nitrogen-fixing bacteria associated with roots; or negative to the plant due to bacteria and fungi which are in concurrence for the same nutritional elements or pathogens and insects which eat roots.

A large body of knowledge suggests that root exudates may act as messengers that communicate and initiate biological and physical interactions between roots and soil

organisms (Bais et al., 2004; Bais et al., 2006; Walker et al., 2003). Among root exudates are of course flavonoids.

1.2.2 White lupin's rhizosphere as a paradigm

White lupin, *Lupinus albus* L., is an annual species of the genus *Lupinus,* tribe *Genisteae* and family *Fabaceae*. It owes its Latin name "*Lupinus*", literally "little wolf" to the fact that it was believed – wrongly – responsible for the poverty of the soils where it was growing (Weisskopf et al., 2005). The term "albus" refers not only to the color of the flowers (Figure 4A), but also to the fine border of white hairs which surrounds the leaves (Figure 4B). White lupin is used mainly for i) human nutrition, because of the high protein and oil levels in seeds, ii) green manure, improving soil structure, as well as nitrogen and phosphorus contents in poor sandy soils and iii) green forage or seeds introduced as nutrient complements to the diet of ruminants (Huyghe, 1997). White lupin grows more easily on soils of neutral or acidic pH, but in contrast to the other cultivated *Lupinus* species, *L. angustifolius*, *L. luteus, L. pilosus* and *L. mutabilis*, it is also able to cope with calcareous soils (Hinsinger and Gilkes, 1995).

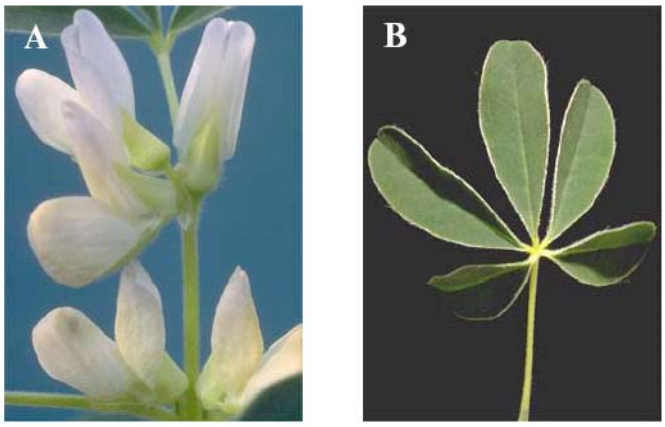


Figure 4. *Lupinus albus L.* **(a)** Flowers. **(b)** Leaf.

Photo: L. Weisskopf.

One of the most remarkable characteristic of white lupin is that it is particularly well adapted to grow on infertile soils containing low amounts of available P, despite belonging to the 20 % of plant species which are not mycorrhized (Dinkelaker et al., 1995; Watt and Evans, 1999). Most of the plant species establish associations with mycorrhizal fungi to deal with phosphate deficiency. This type of fungi is particularly efficient in phosphate recovery, using their hyphae to prospect large volumes of soil. In this symbiosis, plants exchange photosynthates for phosphate and other nutrients (Chabot et al., 1996; Gyaneshwar et al., 2002; Singh and Kapoor, 1999). White lupin, in response to P starvation, develops special root structures, called proteid roots or cluster

roots (Gardner et al., 1981; Purnell, 1960). Cluster roots induce a set of chemical modifications in the rhizosphere aimed at the mobilization and exploitation of sparingly soluble sources of P in soils, mainly by consistently excreting organic acids, protons, phenolics, phosphatase and nuclease (Lamont, 2003; Neumann and Martinoia, 2002; Shane and Lambers, 2005). Therefore, along with mycorrhizae and N_2-fixing nodules, cluster roots are postulated to be the third major adaptation for nutrient acquisition in terrestrial vascular plants (Skene, 2000).

Cluster roots

More than 100 years ago, in the Leipzig Botanical Gardens, Engler described an unusual root structure in the Proteaceae family. He portrayed it as an extensively branched root system covered with long, densely grouped absorption hairs (Skene, 1998). Only 60 years later, Purnell named this special root morphology, described as a root with "dense clusters of rootlets of limited growth", with the term of "proteoid root" after she examined many species of the Proteaceae family (Watt and Evans, 1999). Lamont introduced later the name of cluster root as this root structure does not occur exclusively in this family. It has been reported that it is present in all genera of the family Proteaceae, except the primitive Persoonioideae. Similar structures are present in many others species from the following families: Betulaceae, Casuarinaceae, Cucurbitaceae, Cyperaceae, Eleagnaceae, Fabaceae, Moraceae, Myricaceae and Restionaceae (Skene, 2000).

The factors inducing the formation of cluster roots and the morphogenesis itself are largely unknown. Several reports suggest that the internal phosphate status is the main trigger for cluster root initiation, because foliar application of P reduces the number of clusters (Dinkelaker et al., 1995) and since split root experiments demonstrate that the P status in the shoot is directly correlated with the formation of cluster roots (Neumann and Martinoia, 2002). Recent experiments showed that this is only true for white lupin (Shane et al., 2003b), while in *Hakea prostata* only local conditions trigger the formation of cluster roots (Shane et al., 2003a). Iron deficiency promotes the formation of cluster roots, but not in all species (Neumann and Martinoia, 2002).

Other local factors enhance the initiation of cluster root production. Nutrient-rich patches stimulate it, and cluster roots generally occur in the organic rich upper layer of the soil (Neumann and Martinoia, 2002). Also the presence of microorganisms and their excretion of plant growth factors seem to increase the number of cluster roots (Dinkelaker et al., 1995; Lamont, 2003). One of these factors is auxin or an auxin-like

molecule. In fact, auxin is known to induce cluster root formation even under non-starving conditions (Gilbert et al., 2000; Skene and James, 2000) while cytokinin, an auxin antagonist, inhibits the initiation (Neumann et al., 2000). Nevertheless, the mechanism leading to the initiation and to the coordinated formation of cluster roots are still largely unknown. The capacity to produce proteoid roots appeared several times independently in the evolution of plants and hence it is likely that only few factors are responsible for their production.

Cluster root developmental stages

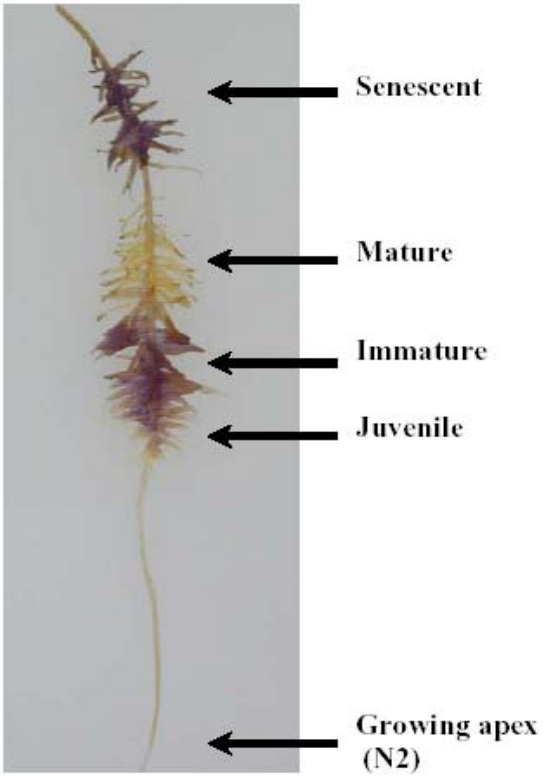


Figure 5. Cluster root stages in white lupin. Four stages can be differentiated in white lupin cluster roots grown in hydroponic conditions using a pH indicator.

In white lupin, formation of cluster roots follows a well-defined developmental pattern (Figure 5) with different stages which last for only few days (Massonneau et al., 2001). Young, growing cluster roots release mainly malate and only low amounts of citrate. Immature cluster roots are fully grown and secrete little amounts of citrate and malate. The mature stage of cluster roots is characterized by a high exudation of citrate and a concomitant acidification of the root environment. Finally, at the senescent stage, the pH increases again and secretion of organic acids stops. All these stages can easily be differentiated in hydroponically grown plants.

The adavantages of cluster roots

The presence of these relatively short (7 mm long) rootlets is sufficient to increase the surface area per unit length of a parent root by over 140×, mainly due to the contribution made by the root hairs. The volume of soil explored within the confines of these rootlets is increased by 288× that of an equivalent length of a nonproteoid root. On an equal mass basis, these proteoid roots have 16 fold larger surface area and explore 33 fold as much soil volume as non-proteoid roots (Lamont, 2003). Moreover, in roots there are zones of high transport and of low transport activity (Skene, 2003). There are zones of the roots where there is a higher concentration of transporters in the plasma membrane which excrete and which take up solutes. These zones are mostly found just after the apices and are the hotspots of root-rhizosphere exchanges. The cluster roots morphology with many concentrated short rootlets appears to be the most adapted structure to maximize the quantity of high transport surface in the minimum volume and quantity of roots. However a limitation of this bottlebrush-like morphology is that the rootlets are so close that the zones of depletion are overlapping. In other words, each rootlet of a cluster root competes with other rootlets for the same nutritional space of the soil. But we should keep in mind that the cluster root-forming species have a different strategy to recover sparingly available nutriments, principally phosphate, than most other plants. The "classical" strategy is to search for free phosphate in a large volume of soil. These plants produce long lateral roots and/or form mycorrhizal associations. In this symbiosis the fungus invades a large volume of soil and takes up phosphate. Cluster root forming species adopt a different strategy: they try to hydrolyze organic phosphate and to solubilize phosphate fixed to calcium, aluminium and iron. To succeed, they have to concentrate their effort in a small volume of soil, where they modify the rhizosphere chemically, mainly by excreting large amounts of organic acids, protons, phenolics and phosphatases. These excretions are limited in space but also in time, at least for the release of citrate and protons. The cluster roots are also ephemeral roots; in white lupin they are active only for a few days. Possible reasons of such a short livespan are i) the burst of proton and citrate excretion is lethal ii) the efficiency and the overlapping uptaking zones render these roots obsolete when their rhizosphere is depleted iii) most of the cluster root-forming species occur in highly seasonal Mediterranean climates where the period of grow is confined to the wet (winter–spring) season (Lamont, 2003).

Secretion physiology and P uptake mechanisms

The phosphate solubilization mechanisms in cluster roots are summarized in Figure 6.

Most of the phosphorus in soil is present as organic phosphate or insoluble mineral phosphate, neither of which can be absorb by plants. Organically bound phosphate, which may account for up to 80 % of the total phosphorus, is mineralized to inorganic phosphate through the release of phosphohydrolases into the rhizosphere (Li et al., 1997; Miller et al., 2001).

Most of the inorganic phosphate is bound to Ca or Fe/Al-humic complexes. Solubilization of Pi from these complexes is partially accomplish by rizoshpere acidication (a wide spread response to P deficiency), and mainly by excretion of carboxylate chelators and phenolic compounds. The latter strategy is restricted to a limited number of plants species, namely the members of the *Proteaceae*. The organic anions (mainly citrate and malate), which are secreted with a concomitant release of protons (Sas et al., 2001), solubilize Pi by ligand exchange, taking the place of Pi in the soil matrix. Gerke et al. and Braum showed that organic anions can desorb Pi from Fe/Al complexes by chelation of the metal cation to which Pi was bound, thus liberating Pi and suppressing its reabsorption (Braum and Helmke, 1995; Gerke et al., 1994).

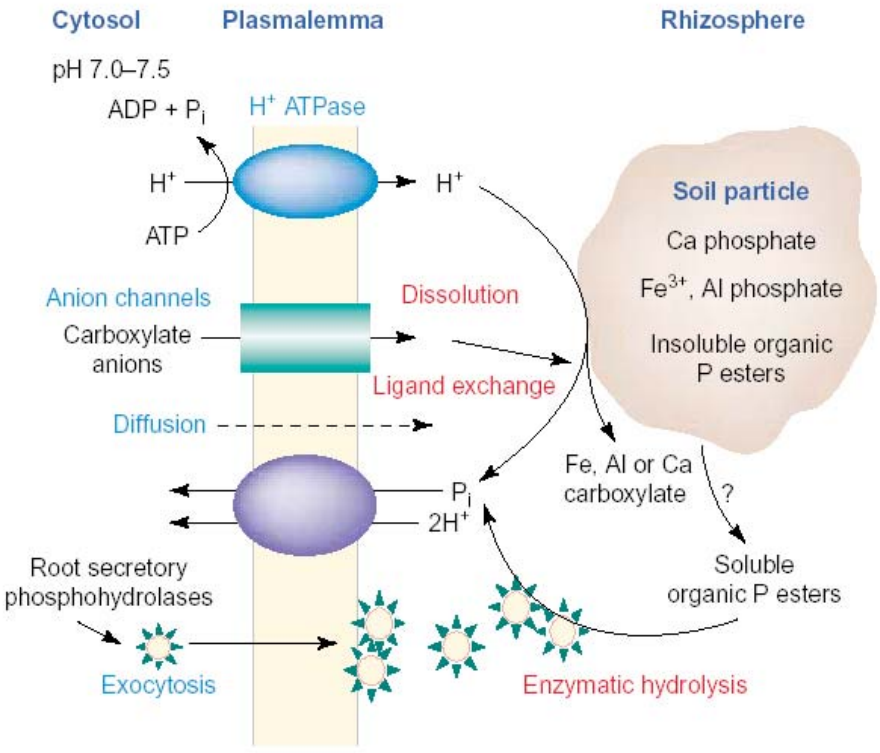


Figure 6. P uptake by cluster roots. Model for root-induced chemical phosphate mobilization in the rhizosphere by exudation of carboxylates, protons and root secretory phosphohydrolases. From Neumann and Martinoia, 2002.

White lupin's rhizosphere

In addition to the general rhizosphere characteristics which I have described above, white lupin's rhizosphere, and especially the cluster root environment, offers very particular conditions to the surrounding microflora. As already mentioned, white lupin's cluster roots go through different developmental stages, which are characterized by different secretion activities: at the juvenile stage, roots are still growing and secrete little amounts of malate and high quantities of isoflavonoids. A few days later, cluster roots reach the immature stage, where isoflavonoid secretion is highest ('phenolic burst'), and organic acid secretion is low. The opposite is observed at the mature stage, characterized by the secretion of very high amounts of citrate and a concomitant release of protons causing rhizosphere acidification; at this stage isoflavonoids secretion drops off. At the senescent stage, the secretion of both isoflavonoids and organic acids decreases strongly. Each cluster root stage is lasting for only few days and this gives an idea of the strong and fast changes which are occurring in the rhizosphere of a growing cluster root: not only in terms of secreted organic compounds, phenolics and organic acids, but also in the physicochemical properties of the rhizosphere, like the pH and the oxygen levels (Neumann et al., 2000), two parameters which are likely to influence the microbial communities (figure 7).

The precise knowledge of the secretion physiology which characterizes each cluster root growth stage makes white lupin a suitable model system to study rhizosphere plant-microorganism interactions and in particular the role of exudated isoflavonoids as extra-signal molecules. During my PhD work I focused my attention on the investigation of the possible role of isoflavonoids as potential protection mechanism of white lupin against microbial degradation of the phosphate-chelating agents (section 4.2).

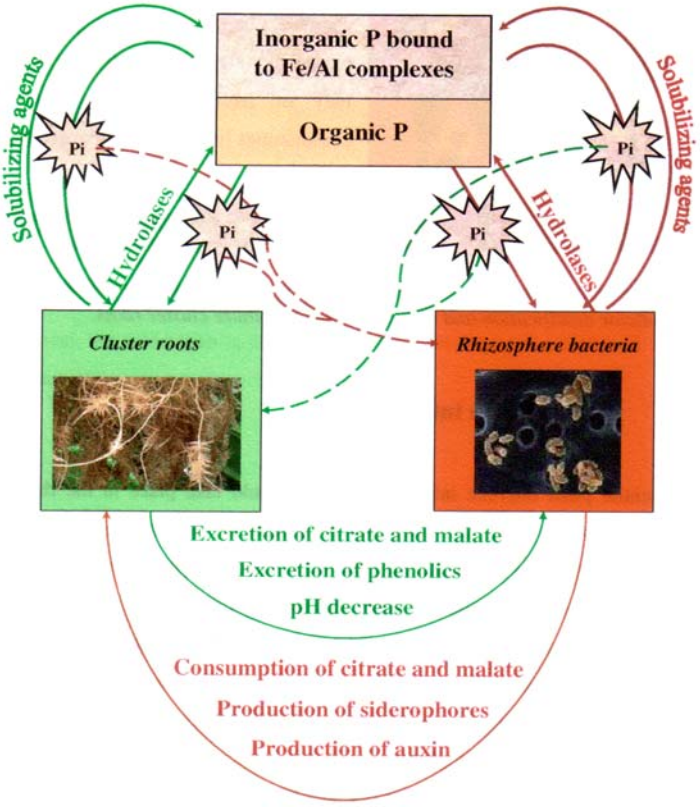


Figure 7. Potential plant-microbe interactions in the rhizosphere of white lupin cluster roots. From Weisskopf, 2006, PhD thesis.

Isoflavonoids exudation from white lupin roots

In contrast to the secretion of organic acids, which has received a lot of attention during the last decades (Dakora and Phillips, 2002; Dinkelaker et al., 1989; Gerke et al., 2000; Gerke et al., 1994; Jones and Darrah, 1994; Ryan et al., 2001b), other components of cluster root exudates have been much less studied. Based on previous indications (Neumann et al., 2000) that phenolics might be secreted in higher amounts in cluster roots than in non cluster roots, we decided to analyze the isoflavonoids produced in and secreted from cluster and non cluster roots, with a detailed comparison of the evolution of isoflavonoid secretion and contents during the different stages of cluster root development. These results are summarized in section 4.1. Except for white lupin, where phenolics have been reported to be more highly secreted in cluster roots (Neumann et al., 2000) and especially present in high amounts in iron deficient plants as compared with phosphate-deficient plants (Hagstrom et al., 2001), no information is available about phenolic secretion in other cluster rooted species. In white lupin, as described above,

isoflavonoids have been previously characterized in detail in the shoots and in the roots (Ingham et al., 1983; Katagiri et al., 2000; Stobiecki et al., 1999; Tahara et al., 1989). However, these studies were never conducted in P deficient conditions and consequently, cluster roots were not investigated. Many analyses were carried out after methanol extraction of ground tissues, without taking into account the root secretion of these compounds. Just recently, reports on secretion of phenolics by white lupin started to appear in the literature (Jung et al., 2003; Pislewska et al., 2002), except for studies dealing with elicitation, where secreted phenolics were taken into consideration earlier.
The available data about white lupin's isoflavonoids published so far gave us a useful start for the setup of the HPLC analysis, but we soon realized that the isoflavonoid profiles we obtained from cluster roots were very different from the profiles described in the literature. The putative reasons for this observation are discussed in detail in section 4.1, but the fact that most likely accounts for this discrepancy is the use of different cultivars of white lupin.

Flavonoids as extra signal molecules

Isoflavonoids have been reported to be involved both in plant nutrition (Dinkelaker et al., 1995; Dixon and Paiva, 1995) and plant microbe interactions (Dakora and Phillips, 1996; Paiva, 2000), acting as multiple cues for both beneficial and pathogenic microorganisms. Phytoalexins, of which isoflavonoids form a major part, are traditionally considered the compounds used in defense. However, some isoflavonoids compounds that classically inhibit pathogens, also serve as chemoattractants, promoters of microbial growth and inducers of nodulation genes in *Rhizobium* bacteria, pointing to a complex picture of isoflavonoid biological role in plant-microorganism interactions. For example, the isoflavonoid pterocarpans, maackiain and pisatin, act as classical phytoalexins in the interaction between garden pea (*Pisum sativum*) and the fungal pathogen *Nectria haematococca*, and maackiain and pisatin detoxyfing enzymes are fungal virulence factors (Enkerli et al., 1998; Wasmann and VanEtten, 1996).
Many studies report on flavonoids as antifungal compounds (Dakora and Phillips, 1996; Tahara et al., 1994; Weidenborner and Jha, 1997). A flavone found in rice is allelopathic to rice fungal pathogens; quercetin, quercetin 3-methyl either and its glycosides inhibited conidia germination in *Neurospora*, and taxifolin appeared to be an anti-fungal agent in pine (Bonello and Blodgett, 2003; Kong et al., 2004; Parvez et al., 2004).

The production and secretion of secondary compounds involved in plant defense rarely occur on a regular basis, but are more often a response to an environmental challenge through the presence of a pathogen. This phenomenon is called "elicitation" and has been the object of many studies in the last few years. It was also investigated in the case of white lupin, by Wojtaszek and Stobiecki, as well as by Gagnon and Ibrahim, who obtained enhanced isoflavonoid root secretion after elicitation with yeast, chitosan, rhizobiae or copper chloride (Gagnon and Ibrahim, 1997; Wojtaszek and Stobiecki, 1997).

We asked the question whether microorganisms isolated from white lupin's rhizosphere would be affected by the isoflavonoid secretion in white lupin. To test this hypothesis, we perfomed *in vitro* tests to assess the antimicrobial acitivity of the isolated isoflavonoids. The results of this work are summarized and discussed in section 4.2. A possible role of isoflavonoids as antifungal compounds in white lupin's rhizosphere is also discussed.

1.3 ROOT EXUDATION: WHICH TRANSPORTERS?

In addition to accumulating biologically active chemicals, plant roots continuosly produce and secrete compounds into the rhizosphere (Bais et al., 2001), such as ions, free oxygen and water, enzymes, mucilage, and a diverse array of carbon-containing primary and secondary metabolites (Bertin et al., 2003; Uren, 2000). Root exudates are often divided into two classes of compounds. Low-molecular weight compounds such as amino acids, organic acids, sugars, phenolics, and other secondary metabolites which account for much of the diversity of root exudates, whereas high-molecular weight exudates, such as mucilage (polysaccharides) and proteins, are less diverse but often compose a larger proportion of the root exudates by mass (Walker et al., 2003). The ability to secrete a vast array of compounds into the rhizosphere is one of the most remarkable metabolic features of plant roots, with nearly 5% to 21% of all photosynthetically fixed carbon being transferred to the rhizosphere through root exudates (Marschner, 1995). Generally, secreted compounds have been shown to vary in

quantity and type, depending on the plant species, the developmental stage, the interacting organism, and a wide variety of environmental factors (Bais et al., 2006).

Although the functions of most root exudates have not been determined, several compounds present in root exudates play important roles in biological processes (Bais et al., 2003; Bais et al., 2002; Kneer et al., 1999). Through the exudation of a wide variety of compounds, roots may regulate the soil microbial community in their immediate vicinity, cope with herbivores, encourage beneficial symbioses, and inhibit the growth of competing plant species (Nardi et al., 2000). Root exudation is also involved in nutrient acquisition, such as Fe, Zn and P (Hopkins et al., 1998; Neumann and Romheld, 1999; Zhang et al., 1991), especially in low-nutrient environments (Dakora and Phillips, 2002). Certain compounds such as phytosiderophores, mugineic acid, and malate improve iron availability (Fan et al., 2001), whereas others, including organic acids, assist increasing the availability of phosphate and micronutrients (as well documented in section 1.2), or relieve Al-toxicity by chelating phytotoxic metals (Heim et al., 2001).

Root exudation can be broadly divided into two active processes. The first, root excretion, involves gradient-dependent excretion of waste materials with unknown functions, whereas the second, secretion, involves exudation of compounds with known functions, such as lubrication and defense (Bais et al., 2004; Uren, 2000). Roots release compounds via at least two potential mechanisms: active transport across the cellular membrane or release from root border cells and root border-like cells, which separate from roots as they grow (Hawes et al., 2000; Vicre et al., 2005).

1.3.1 Cellular mechanisms of root exudation

Root exudates are released in four different major ways from living root system: diffusion, ion channel, vesicle transport and ATP-dependent transport. Low molecular weight organic compounds such as sugars, amino acids, carboxylic acids, and phenolics could be released using a passive process involving steep concentration gradients between the cytoplasm of intact root cells (millimolar range) and the soil (micromolar range). However, the possibility of direct diffusion through the lipid bilayer of the plasmalemma strongly depends on membrane permeability, determined by the physiological state of the root cell and polarity of the compounds to be exuded. Permeation of lipophilic exudates is generally facilitated by this method. Under a typical cytosolic pH of approximately 7.1–7.4, polar intracellular low-molecular weight

compounds, including amino and carboxylic acids, exist as anions with low plasmalemma permeability. Thus, for these compounds other mechanisms of release are required. The large cytosolic K^+ diffusion potential (Samuels et al., 1992) and the extrusion of protons through ATPase generate a positive charge gradient which promotes not only the uptake of cations from external solutions but also the outward diffusion of carboxylate anions.

Very little is currently known about the molecular mechanisms for the trafficking of phytochemicals. Many of the metabolites that the cell synthesizes show cytotoxic activities that would prevent their accumulation in the cytoplasm. Evidences showing the presence of intracellular bodies in plant cells accumulating large quantities of secondary metabolites (Facchini, 2001; Grotewold, 2001), support the idea of phytochemicals transport from the site of synthesis to the site of storage by vesicles. Although the mechanisms by which these compounds are transported from the ER to the plasma membrane are not known, it is possible that they are transported by ER-originating vesicles that fuse to the cell membrane and release their contents to the soil. Vesicles with the above-described properties and containing green autofluorescent compounds have been identified in maize cells ectopically expressing the P regulator of 3-deoxy flavonoid biosynthesis (Grotewold et al., 1998). The vesicles fuse and form large green fluorescent bodies that migrate to the surface of the cell and fuse to the cell membrane and release the green fluorescent compound to the cell wall. Interestingly, the accumulation of the green fluorescence in the cell wall is increased by treatment with Golgi-disrupting agents, such as brefeldin A, suggesting a trans-Golgi network-independent pathway for the secretion of these compounds.

Besides the mechanisms described above, membrane transporters such as ABC and MATE transporters might be also responsible for the secretion of pythochemicals. So far, very little is known about their involvement in root exudation and only a plasma membrane H^+-ATPase and ion channels have been proposed in wheat, lupin and soybean to account for the secretion of malate, citrate and oxalic acid respectively (Shen et al., 2005; Yan et al., 2002). However, working on the assumption that plasma membrane ABC transporters might be involved in the secretion of defense metabolites, and their expression may be regulated by the concentration of these metabolites, Jasinski et al. identified a plasma membrane ABC transporter (NpABC1) from *Nicotiana plumbaginifolia* by treating cell cultures with various secondary metabolites. Interestingly, addition of sclareolide, an antifungal diterpene produced at the leaf surface of *Nicotiana* spp., resulted in the expression of NpABC1 (Jasinski et al., 2001). These findings suggest

that NpABC1 and likely other plasma membrane ABC transporters have a role in the secretion of secondary metabolites involved in plant defense. Very recently a pharmacological approach provided evidences that root secretion of certain secondary metabolites is indeed an ATP-dependent process, and suggested that ABC transporters, among other transporters, could be implicated in this process (Loyola-Vargas et al., 2007). A general overview about the involvement of ABC transporters in membrane transport of endogenous secondary metabolites in plant and of those mediating the transport of plant products in heterologous systems has been given by Yazaki (Yazaki, 2006). In figure 8, membrane transport of secondary metabolites and involved ABC transporters are depicted.

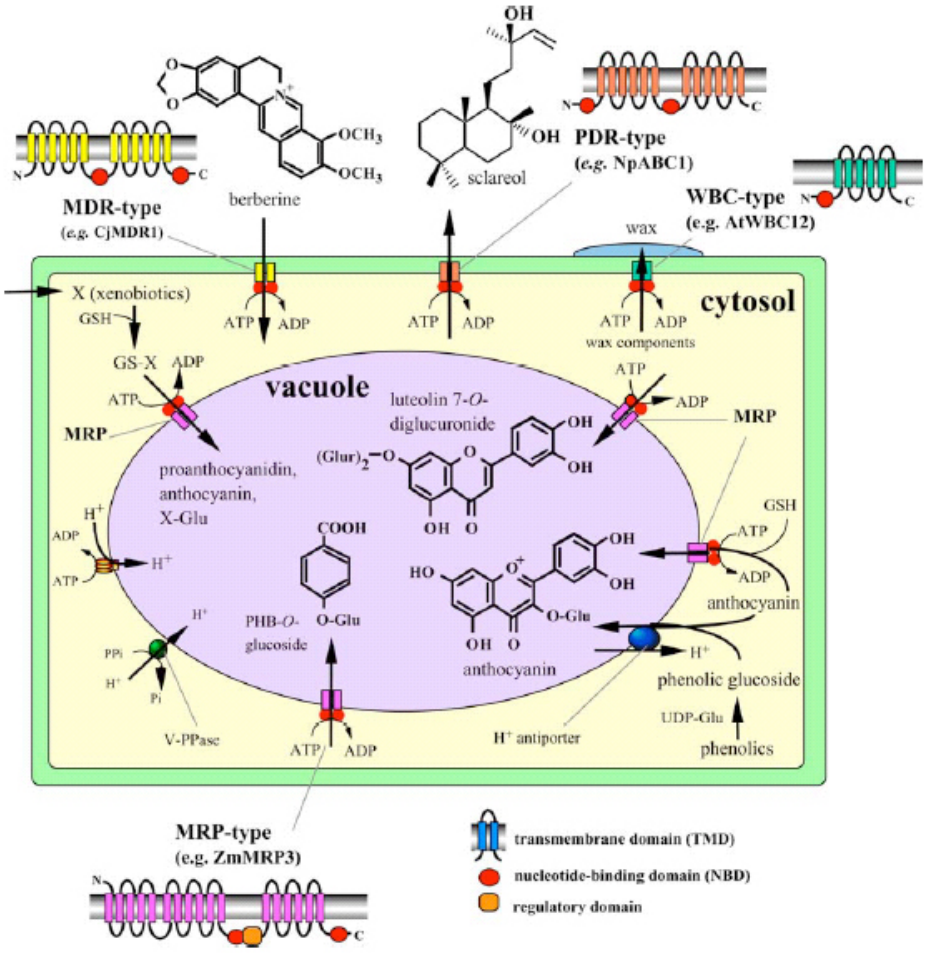


Figure 8. Scheme of membrane transport of secondary metabolites and ABC transporters involved. Representative natural products, which are proposed to be transported by plant ABC transporters, are indicated. The typical topology of each ABC transporter subfamily is also shown. From Yazaki, 2006

Based on this information and the literature implicating H^+-ATPase and anion channels in root sectretion, I carried out a survey of potentially involved transporters in the secretion of secondary compounds. In collaboration with Professor Vivanco from the Colorado State University we provided a first comprehensive examination of the role of ABC transporters in root exudation; obtained results and discussion are presented in section 4.4. Within the frame of my PhD work, I also investigated the possible role of two MATE genes isolated from white lupin under phosphate starvation as possible candidates as phenolics and/or organic acids transporters. In section 4.3 I summarized the preliminary results I obtained and also discussed the difficulties I encountered during the molecular experiments with a plant like lupin, for which only a limited genetic knowledge is available. In the following paragraphs I will give a short description about the multigene families of ABC and MATE transporters.

1.3.2 ABC transporters

The ATP-binding cassette (ABC) transporter family is one of the largest membrane protein families. ABC transporters are widespread in the phylum of Procarya (Archea and Bacteria) and Eucarya. Each phylum possesses many ABC transporters subfamilies. The ABC proteins are implicated in transporting a large variety of substances like drugs, amino acids, various nutrients or peptides against the concentration gradient using MgATP as energy source. Certain ABC proteins recognise and transport several chemically different compounds but many of them are relatively specific.

ABC transporters share a common basic structure formed by the repetition of two structural domain: a cytosolic domain (nuocleotide bindind domain, NDB), which contains conserved sequences involved in ATP binding and hydrolysis, and a hydrophobic trans-membrane domain (TMD), involved in translocating and possibly binding the substrate. These general properties have been extensively reviewed (Higgins, 2001). The NDB and TMD domain can be expressed independently, as in many prokaryotic ABC proteins. For many eukaryotic ABC proteins, the domains are fused to generate proteins consisting of either one NBD and one TMD ("half size") or two NDBs and two TMDs ("full size") sometimes with additional TMDs (Higgins, 2001; Holland and Blight, 1999). The full-size ABC transporters can be subdivided into three families: mutidrug resistance (MDR, $[TMD\text{-}NBD]_2$), multidrug resistance-associated protein (MRP, $TMD_0\text{-}[TMD\text{-}NBD]_2$), and pleiotropic drug resistance (PDR, [NBD-

$TMD]_2$). These names come from the initial observation that members of these families confer resistance to various drugs. However, these definitions are now too restrictive, as these proteins transport also other substrates and are involved in functions other than detoxyfying cells (Martinoia et al., 2002).

Only two plant ABC transporters were reported in 1992 (Dudler and Hertig, 1992; Higgins, 1992). Since then, the interest for ABC transporters in plant has increased and many new genes were described. General overviews of plant ABC transporters were published first in 1998, then in 2000; in 2001, a systematic inventory of the ORFs encoding ABC proteins found in the *Arabidopsis thaliana* complete genome was done (Rea et al., 1998; Sanchez-Fernandez et al., 2001; Theodoulou, 2000). Almost two years after those reports, a whole chapter about plant ABC transporters was included in the book: 'ABC proteins: from Bacteria to Man' (Rea et al., 2002).

At present, the *A. thaliana* genome encodes 131 ABC proteins and at least 103 of them are membrane proteins (Martinoia et al., 2002; Sanchez-Fernandez et al., 2001). Interestingly, the number of *Arabidopsis* full-size ABC transporters exceeds those reported in yeast (Decottignies and Goffeau, 1997) and in humans (Dean and Allikmets, 2001), suggesting that the sessile life of plants requires a large range of adaptations and that the higher number of potential substrates (secondary metabolites) produced by plants compared to other organisms (Dixon, 2001) may need a large number of transporters. On the other hand, because of several ABC transporters are the result of gene duplication events within the *Arabidopsis* genome during evolution, some functional redundancy might also exist.

MDR subfamily

PGPs were first identified in mammalian cancer lines because their overexpression confers MDR to chemotherapeutic cancer treatments (Ambudkar et al., 2003). In non-cancerous mammalian tissues, with the exception of ABCB8, which is apparently localised to mitochondrial membranes, PGPs are localised to the plasma membrane where they are thought to function as detoxifying efflux pumps. Plant PGPs were initially presumed to function in dexotification, but they where later shown to have rather a developmental role. *AtPGP1* was the first plant ABC transporter gene cloned (Dudler and Hertig, 1992). In *A. thaliana,* 22 other homologues have been identified. Together they constitute the second largest subfamily of ABC protein in this plant. To date, the detailed characterisation of only two *Arabidopsis* PGPs (AtPGP1 and AtPGP4

(Geisler et al., 2005; Noh et al., 2001; Terasaka et al., 2005 and this PhD thesis) has been reported in the literature.

MRP subfamily

The observation of multidrug resistance in small cell lung cancer line in which MDR protein expession levels were normal, allowed to identify this subclass of ABC transporters. Multidrug resistance-associated protein (MRP) overexpression and transfection experiments demonstrate that it is a gluthatione (GS) conjugate transporter in mammal. The functional resemblance of transporters responsible of GS-conjugates and a gluthationated herbicide uptake by isolated vacuoles and vacuolar membrane vesicules from plants orientated the studies of the 15 MRP homologues on vacuolar membrane (Lu et al., 1998; Martinoia et al., 1993; Martinoia et al., 2000). The MRP transporters are known to function in vacuolar sequestration of glutathionylated compounds, malonylated chlorophyll catabolites and glucuronides (Klein et al., 2000; Klein et al., 1998; Martinoia et al., 2002; Rea et al., 1998; Theodoulou, 2000) and in guard cell ion flux (Klein et al., 2003).

PDR subfamily

The PDR family is found only in fungi and plants. It was first characterized in the yeast *Saccharomyces cerevisiae* (Bauer et al., 1999; Decottignies and Goffeau, 1997), in which PDRs were shown to confer resistance to a large set of functionally and structurally unrelated toxic compounds (antifungal and anticarcer drug) (Rogers et al., 2001). In the *Arabidopsis* genome, 15 PDR genes have been annotated and their organization and physiological roles have just been reviewed (Crouzet et al., 2006). The PDR subfamily members are characterized in fungal systems as efflux transporters of cytotoxic compounds (Balzi et al., 1994). In plants, they are involved in exporting antifungal diterpene defense compounds to the leaf surface (Jasinski et al., 2001; Stukkens et al., 2005) and also in disease resistance (Kobae et al., 2006).

1.3.3 MATE transporters

The multidrug and toxic compound extrusion (MATE) family of putative secondary transporters is unique to plants and microbes (Brown et al., 1999). The MATE protein family members are thought to encode efflux proton antiporters (Morita et al., 1998), whose transport function requires H^+-ATPase and pyrophosphatase activity for maintenance of a proton gradient across the membrane.

MATE proteins in prokaryotes, as exemplified by the NorM multidrug resistance protein from *Vibrio parahaemolyticus* and its homolog in *E. coli*, YdhE, mediate resistance against diverse drugs including fluoroquinolone antibiotics (Morita et al., 1998). Functionally, NorM has been described as a Na^+-driven multidrug efflux pump (Morita et al., 2000). *MATE1*, one of two orthologous genes in humans, encodes a transporter mediating H^+-coupled cellular efflux of toxic organic cations such as tetraethylammonium, and thus represents the polyspecific organic cation exporter that transports toxic organic cations into urine and bile (Otsuka et al., 2005).

So far, 56 MATE proteins have been identified in the *Arabidopsis* genome. Five MATE genes have been initially characterized by mutant analysis. They are involved in lateral root formation (ALF5, AtDTX19, Diener et al., 2001), iron homeostasis (FRD3, AtDTX43, Rogers and Guerinot, 2002) or disease resistance (EDS5, AtDTX47, Nawrath et al., 2002). Complementation of the norfloxacin-hypersensitive *E. coli* strain KAM3, lacking the multidrug efflux pump AcrAB, resulted in functional cloning of *AtDTX1* (Li et al., 2002). Although biochemical data on transport properties and functional aspects of family members are scarse, the *AFL5* (Diener et al., 2001) and *TT12* (Debeaujon et al., 2001) mutant phenotypes are consistent with the involvement of these genes in transporting small, organic molecules. The *AFL5* gene is expressed in *Arabidopsis* root epidermis and when mutated leads to increased root sensitivity to a variety of inhibitory compounds, suggesting that *AFL5* transports these inhibitory compounds either out of the epidermal cells or into the vacuole. The *TT12* mutant phenotype is an alteration in seed coat pigmentation. It has been postulated that the *TT12* protein may control the vacuolar sequestration of flavonoids in the seed coat endothelium. Recently a study done in our laboratory, provided biochemical evidence that TT12 expressed in yeast is responsible for vacuolar flavonoid/H^+ transport (Marinova and Klein, submitted), leading to the proposition that members of the MATE family in plants are involved in flavonoid deposition either in the vacuole or in the apoplastic space depending on their subcellular localization.

1.4 ROLE OF FLAVONOIDS IN PLANT DEVELOPMENT

In a recent review, Peer and Murphy (2006) suggested that intra-organismal flavonoid signalling is probably a by-product of the evolution of plant signalling mechanisms, in an environment where flavonoids are present for purposes of extra-organismal signalling and defense, and a role in initial protection from oxidative stress. Nevertheless, development of molecular biological tools and high throughput metabolic profiling technologies have provided new opportunities for the identification of specific and non-specific sites of flavonoid regulation, suggesting that these compounds maybe involved in undiscovered important action as internal signals within the intact plants.

In the section 1.1.3, I have described the multi biological roles of flavonoids both as exudated molecules and internal signals. In this section I will first briefly discuss the potential targets of flavonoid regulation at the molecular level, and then focus on the effect of flavonoids on auxin transport, as major pathway studied during my PhD work.

1.4.1 Flavonoids as intra signalling molecules

A large body of knowledge exist about flavonoids in cellular signal transduction and transport pathways, with potential targets ranging from transcription factors and kinases to ATP-binding cassette transporters and aminopeptitades (for a detailed review see Peer and Murphy, 2006).

As already reported in section 1.1.2, flavonoids are found to be localized also in the nucleus, suggesting a possible function of these compounds in transcriptional regulation of endogenous gene expression. Based on experiments on mammalian transcription (Liang et al., 1999; Wadsworth et al., 2001), it is likely that most of this regulation involves inhibition of phosphorylation signalling cascades or specific kinases. Flavonoids, especially flavonols, have been shown to interact with proteins *in vivo*, and the interaction depends on B-ring substitution pattern of the interacting flavonoids (Marko et al., 2004). Several targets have been identified in *Arabidopsis*. Among them, PINOID (PID), a serine/threonine kinase; ROOT CURL IN NAPHTHYLPHTHALMIC ACID1 (RCN1), the subunit A of protein phosphatase 2A (PP2A); and multiple drug-resistance/P-glycoprotein (MDR/PGP) ABC transporters have been shown to be flavonoid targets.

PID is sensitive to NPA, has a role in polar auxin transport (Benjamins et al., 2001), and it has been shown to colocalize with flavonoids. Analysis of the localization of PIN auxin efflux facilitator proteins and auxin transport in flavonoid-deficient mutant backgrounds suggests that PID-mediated kinase activity may be modulated by endogenous flavonols (Peer et al., 2004).

RCN1 was isolated in a screen for mutants with altered responses to NPA (Garbers et al., 1996). RCN1 is essential for PP2A activity and *rcn1* mutant has auxin-related phenotype and increased sensitivity to okadaic acid, a phosphatase inhibitor (Deruere et al., 1999; Garbers et al., 1996). Like PID, RCN1 may regulate polar auxin transport via a mitogen-activated protein kinase (MAPK) activity (DeLong et al., 2002), which has been shown to be affected by flavonoids in other systems (Lin et al., 2004).

In mammals, flavonoids alter the function of PGPs by binding to ATP binding sites or by altering the conformation of the protein (Castro et al., 1999; Ferte et al., 1999). Flavonol inhibition of PGP activity in *Arabidopsis* has recently been shown in our laboratory by Geisler et al. (2005).

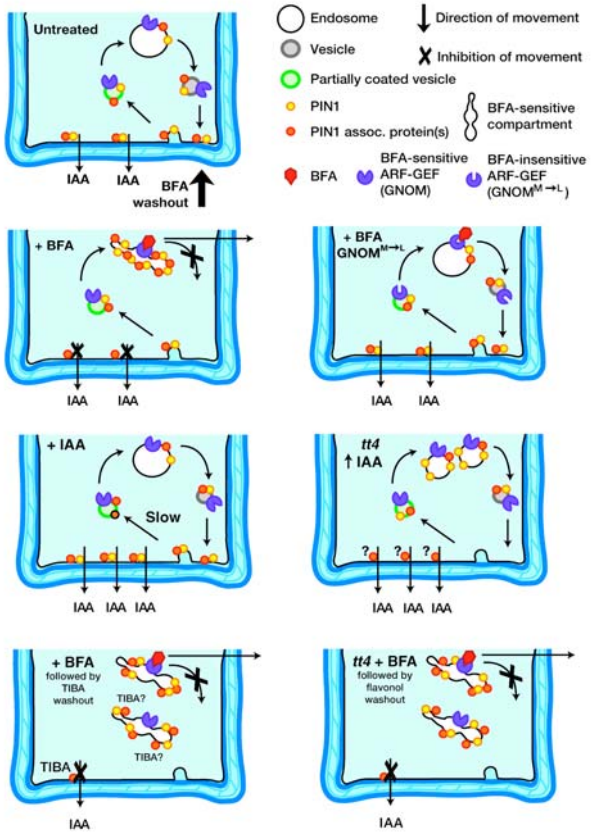


Figure 9. Flavonols and the BFA-induced compartmentalization.

Flavonols can interact with vesicular transport in tissues exhibiting brefeldin A (BFA) sensitive trafficking (Peer et al., 2004); BFA is a lactone antibiotic from *Penicillium*

brefeldianum that is used to inhibit protein secretion. The auxin efflux facilitator protein PIN1 has been shown to traffic to the plasma membrane through BFA-sensitive compartments via a mechanism that is also sensitive to the auxin efflux inhibitors triiodobenzoic acid (TIBA) and NPA (Geldner et al., 2001). When flavonoid-deficient *tt4* *Arabidopsis* mutants were treated with flavonols, PIN1 was irreversibly retained in BFA-sensitive compartments (Peer et al., 2004). The effect was reversible in wild type, suggesting indirect flavonol modulation of trafficking in cells where they accumulate, but direct interference with trafficking in cells not conditioned to their presence (Peer et al., 2004) (Figure 9). The running hypothesis is that flavonoids may alter the activities of proteins required for trafficking by binding them directly or altering their phosphorylation states.

1.4.2 Auxin and polar auxin transport

Charles Darwin, studying the response of canary grass coleoptiles to unilateral light, first demonstrated the existence of a moving signal that mediated plant phototropism. This discovery of the first plant hormone, later termed auxin (from the Greek "auxein", which means to grow), was followed several decades later by the identification of auxin as indole-3-acetic-acid (IAA) (Koegl and Kostermans, 1934; Went and Thimann, 1937), which is now considered the most common bioactive form. Sinthetic plant regulators such as 1-napthylacetic acid (1-NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) also display auxin-like activities, and are commonly used in auxin bioassays (Figure 10).

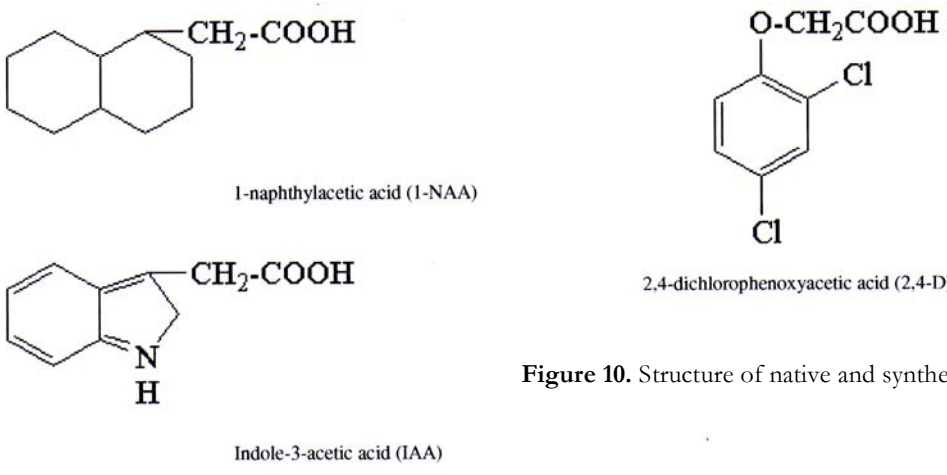


Figure 10. Structure of native and synthetic auxins.

Auxin metabolism

Auxin is thought to be synthetized in plants locally, in young growing regions, predominantly in the shoot apex, young leaves, and developing seeds (Ljung et al., 2001), but it seems that almost any plant tissue can at certain times be responsive to auxin (Davies, 1995). Two different biosynthetic pathways are involved in IAA biosynthesis, one which uses tryptophan as a precursor, and another, discovered about ten years ago (Ljung et al., 2002; Wright et al., 1991), which bypasses tryptophan and uses indole as a precursor for IAA biosynthesis. Recently, using gain-of-function approaches in *Arabidopsis*, redundant pathways have been defined within the tryptophan-dependent pathway (Zhao et al., 2001). These results reveal a complex situation where two genetically discrete pathways can convert tryptophan to auxin, both starting from the same precursor and result in the formation of the same signal messenger. However, the details of auxin biosynthesis and its regulation remain largely elusive. One interesting observation is that pathways involved in the biosynthesis of indolic compounds are closely related to stress, wounding and pathogen attack. So it might be expected that IAA biosynthesis would also be affected by such environmental signals, suggesting a strong link between IAA metabolism and plant success in adapting to its environment.

Cellular auxin levels in a specific tissue (IAA homeoastasis) result from complex interplay between auxin synthesis, conjugation, and transport (Ljung et al., 2005). Free IAA is active at low concentrations (10^{-6}-10^{-8} M), while higher concentrations can be toxic to the plant. On average, 95% of all IAA in a plant is found in a conjugated form (Ljung et al., 2002). During conjugation the side-chain of the active hormone is modified, and two distinct groups of conjugates have been described in a variety of plant species: (1) ester-type conjugates, in which the carboxyl group of IAA is linked via a hydroxyl to sugars (for example glucose) or cyclic poly-ols (like inositol), and (2) amide-type conjugates in which the carboxyl group forms an amide (e.g. peptide) bond with amino acids or polypeptides (Normanly and Bartel, 1999; Seidel et al., 2006). These IAA-conjugates are thought to be involved in a variety of hormonally-related processes: a) in the transport of IAA within the plant; b) the storage and subsequent release of IAA; c) protection of IAA from enzymatic destruction; d) as components of a homeostatic mechanism for control of IAA levels; and e) as an entry route into the subsequent catabolism of IAA. Using such mechanisms, a plant is able to change its hormone levels and thus control its growth and development under changing environmental conditions.

Polar auxin transport (PAT)

Already in 1920s, Cholodny and Went were independently trying to understand how auxin moves from the apex, where it is synthesize, into the elongation zone (Went, 1974). They hypothesized for the first time that auxin is transported in a polar fashion from cell-to-cell along the apical-basal plant axis. Nevertheless, molecules and mechanisms driving this transport remained unknown for the next 70 years. Today, thanks to a collection of outstanding publications in the field, we learnt a lot about the physiology and the cellular components of polar auxin transport.

In general, two main pathways describe the transport of auxin in plants: a fast, non-directional transport in the phloem and a slower, directional, polar auxin transport (PAT) in various tissues. Phloem transport occurs in both basipetal and acropetal directions, proceeds relatively fast and seems to be responsible for the transport of assimilates and inactive auxin conjugates (Nowacki and Bandurski, 1980). Experiments in pea showed that the labeled auxin transported within the phloem was later detected in the PAT system indicating that both transport pathways may be linked (Cambridge and Morris, 1996). In contrast to phloem transport, PAT is specific for active free auxins, occurs in a cell-to-cell manner and has a strictly unidirectional character.

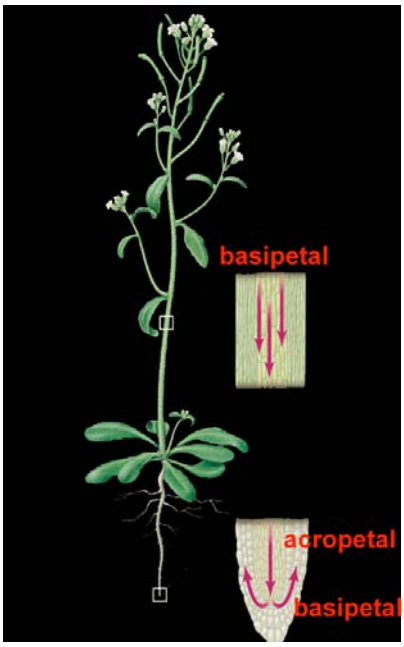


Figure 11. Polar auxin transport (PAT) in plant. Modified from Jones, 1998.

PAT is a central molecular mechanism determing plant polarity, patterning, and growth (see below). The main PAT stream runs from the apex basipetally towards the base of the plant. In shoots, PAT occurs in lateral directions (Morris and Thomas, 1978). In roots, auxin transport is more complex, with two distinct polarities. IAA moves acropetally (towards the root apex) through the central cylinder and basipetally (from the root apex towards the base) through the outer layers of root cells (Jones, 1998; Lomax et al., 1995, Figure 11).

PAT is a process that require energy, is saturable and sensitive to protein synthesis inhibitors. The chemiosmotic hypothesis, postulated in 1970s and described by two milestones papers (Raven, 1975; Rubery and Sheldrak.Ar, 1974), is still a coherent model to explain the physiology of polar auxin transport (figure 12). IAA is a weak acid with a

dissociation constant of pK = 4.75. In the apoplast (pH ~ 5.5) about 15% of IAA exists in its protonated form (IAAH). This non-charged, lipophilic molecule passes easily through the plasma membrane by diffusion or through the action of a saturable uptake carrier, probably working as an $IAA^-/2H^+$ co-transporter (Benning, 1986; Goldsmith, 1977; Yang et al., 2006). In the more basic cytoplasm (pH ~ 7) IAAH dissociates and hence the resulting IAA^- anion is 'trapped' inside the cell or within vesicular compartments due to its poor membrane permeability; its export therefore requires a specific efflux carrier. The polarity of the auxin flux depends on the asymmetric distribution of influx and efflux plasma membrane carriers.

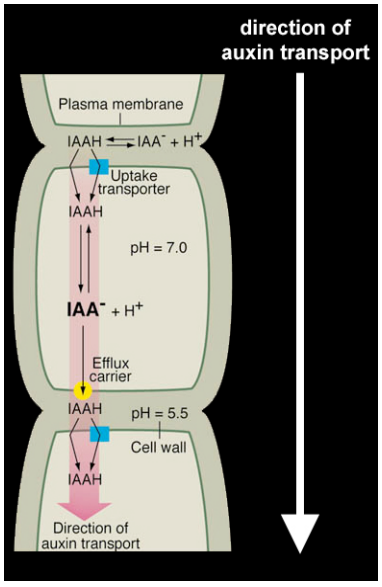


Figure 12. Physiology of cellular polar auxin transport. Modified from Jones, 1998.

Auxin influx and efflux pathways can be physiologically distinguished using auxin efflux inhibitors (AEI), such 1-*N*-naphthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA). AEI have been using as tools for the establishment of the role of the auxin efflux carriers in plant development. The recent isolation of 1-naphthoxyacetic acid (1-NOA) and 3-chloro-4-hydroxyphenylacetic acid (CHPAA) specifically inhibiting auxin uptake in tobacco culture cells (Imhoff et al., 2000), has provided the scientists with a new tool for the study of influx carrier activity in the cell (Parry et al., 2001).

Proteins that mediate polar auxin transport

The identification of PAT components in *Arabidopsis* results from genetic screens for mutants displaying resistance to auxins and ethylene or abnormal rensponses to auxin transport inhibitors. To date, three main different putative classes of auxin carriers have been identified: the influx carrier AUX1 (Bennett et al., 1996; Marchant et al., 1999), the efflux carrier family of PIN proteins (PINFORMED) (Galweiler et al., 1998; Paponov et al., 2005), and more recently members of the PGP subfamily of ABC transporters (Geisler et al., 2005; Geisler and Murphy, 2006; Noh et al., 2001).

The *aux1* mutant, which confers a root agravitropic and auxin-resistant phenotype, was isolated in 1996 by Bennett and co-workers. Since then, several lines of evidences have been collected that support AUX1 function as an auxin uptake carrier. *AUX1* gene

encodes a 485 amino acid protein sharing significant similarity with plant amino acid permeases favouring the role for AUX1 in the uptake of the tryptophan-like IAA (Bennett et al., 1996). The membrane permeable 1-NAA rescues the *aux1* root agravitropic phenotype much more efficiently than the membrane less permeable IAA or 2,4-D and this rescue coincides with restoration of basipetal auxin transport (Marchant et al., 1999; Yamamoto and Yamamoto, 1998). Moreover, this phenotype, including its specific NAA rescue, can be mimicked by growing seedlings on recently isolated inhibitors of auxin influx (Parry et al., 2001). Uptake assays using radioactively labelled auxins and auxin analogues revealed that *aux1* roots accumulated significantly less 2,4-D than wild-type roots (Marchant et al., 1999). Very recently, a functional biochemical characterization of AUX1 3^{H}-IAA uptake acitvity in *Xenopus* oocytes has been provided (Yang et al., 2006). The AUX1 protein is localized in a remarkable pattern in a subset of stele, columella, lateral root cap and epidermal cells exclusively in the root tips (Swarup et al., 2001; Swarup et al., 2004). Disruption of AUX1 causes changes in cell-specific auxin accumulation associated with tissues mediating basipetal auxin transport (Rashotte et al., 2001; Swarup et al., 2001). *aux1* mutants are also defective in auxin supply to the root tip, since mutant root tips contain less free auxin than those of wild type. Localization of AUX1 at the upper side of protophloem cells together with less accumulation of free auxin observed at the root tip of *aux1* mutant, suggest a role of AUX1 protein in unloading of the bulk flow via the protophloem to the root apical meristem (Swarup et al., 2001).

There are eight *PIN* genes in the genome of *Arabidopsis thaliana*, showing close evolutionary relationships (Paponov et al., 2005). The PIN proteins that have been characterized so far demonstrate unique specific expression patterns and are present in particular cell types and cell layers (Friml et al., 2002a; Friml et al., 2002b; Galweiler et al., 1998; Muller et al., 1998). *pin* mutations generally exhibit growth phenotypes that are consistent with the loss of directional auxin transport in the corresponding tissue (Benkova et al., 2003; Blilou et al., 2005; Friml et al., 2003). Although the evidence that PIN proteins do in fact transport auxin was for a long time equivocal, recent data strongly indicate that they are *bona fide* auxin efflux carriers (Petrasek et al., 2006). In this exceptional work, the ability of PIN proteins to mediate auxin efflux from mammalian and yeast cells without needing additional plant-specific factors has been proved. Figure 13 summarizes the localization of PIN proteins and their control of IAA fluxes. Genetic analysis implicates different PINs in various, seemingly unrelated, developmental

processes (Friml et al., 2003). In *Arabidopsis*, PIN1 mediates organogenesis and vascular tissue differentiation (Benkova et al., 2003; Galweiler et al., 1998; Reinhardt et al., 2003), PIN2 root gravitropic growth (Muller et al., 1998), PIN3 shoot differential growth (Friml et al., 2002b), PIN4 root meristem activity (Friml et al., 2002a), and PIN7 early embryo development (Friml et al., 2003).

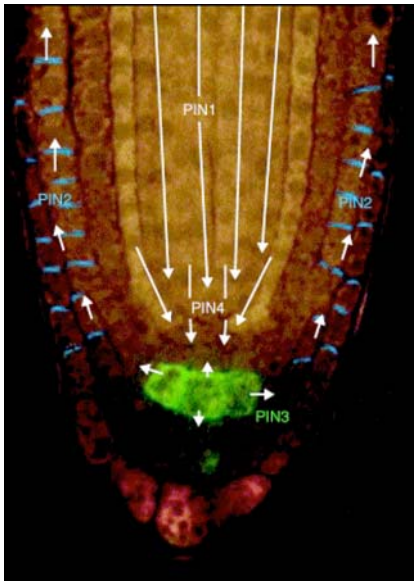


Figure 13. PIN protein localization at the root tip and their control of IAA fluxes. From Friml et al., 2003

However, strong, embryo lethal phenotypes of pin1,3,4,7 quadruple mutants, which contrast with much weaker and often not fully penetrant defects in most of the single pin mutants, suggest a functional redundancy within the PIN gene family (Friml et al., 2003). Moreover, recent analysis of various combinations of *pin* mutants revealed ectopic expression of PIN proteins in some mutant combinations (Blilou et al., 2005; Vieten et al., 2005), but the underlying mechanism and biological importance of this effect is still unclear.

As mammalian PGP orthologs are associated with multiple drug resistance, plant PGPs were initially presumed to function in detoxification. AtPGP1 was the first plant PGP to be identified as part of an effort to isolate proteins that function in broad-spectrum herbicide resistance (Dudler and Hertig, 1992). A role for PGPs in the regulation of auxin transport was only ten years later demonstrated by a combination of genetic and biochemical approaches (Geisler et al., 2005; Luschnig, 2002; Muday and Murphy, 2002; Noh et al., 2001). Knockout mutants in *AtPGP19*/*AtMDR1* and *AtPGP1* genes have phenotypes consistent with altered auxin transport, including epinastic cotyledons and reduced apical dominance. Basipetal auxin transport is reduced substantially in *pgp19* inflorescences and *pgp19* and *pgp1* hypocotyls. The more severe dwarf phenotype of the

double mutant *pgp1pgp19* compared to the single mutants, suggests overlapping, tissue-specific functions also in the PGPs family (Geisler et al., 2003). PGP1 exhibits non-polar expression in small, meristematic cells of the shoot and root apices, while in regions above the apex is localized in a polar, predominantly basal fashion. A strong support for PGPs as auxin transporters came from a recent study in our laboratory, in which PGP1 was shown to catalyze *in planta* and in other heterologous expression systems the primary active export of IAA, 1-NAA, and oxidative IAA breakdown products (Geisler et al., 2005).

The PGPs members characterized so far have been suggested to work as IAA efflux carriers. During my PhD project I had the opportunity to investigate the role of a new member of the PGP subfamily, AtPGP4, which showed to play also a critical role in plant growth and development, most probably acting as an auxin uptake sink in the root cap, driving the basipetal redirection of auxin. These results are summarized and discussed in section 4.5.

IAA homeostasis and plant development

The quantitative temporal and spatial distributions of IAA play important roles in the development of a plant throughout its life cycle. In vascular plants, auxins, primarily indole-3-acetic acid (IAA), regulate gene expression, cell division, cell elongation and differentiation in plant tissue. Auxins also affect embryo and root patterning, vascularization, phototropism, geotropism, apical dominance, fruit development and flower development (Davies, 1995).

During my PhD work, the characterization of *pgp4* mutant phenotype revealed an obvious defect in lateral root and root hairs development. Indeed in literature, genetic and physiological evidences report that auxin is required at several specific developmental stages for root hair (RH) development (Rahman et al., 2002) and lateral root (LR) formation, suggesting a role of PGP4 in the control of plant development.

IAA transported from the developing leaves to the root system is detectable as short-lived pulse in the roots and is required for the emergence of lateral root primordia (LRP) during early seedling development (5-7 dag). However, removal of apical tissues prior to detection of the IAA pulse in the root inhibits LRP emergence, but has a minimal effect on LRP initiation. A basipetal, rather than an acropetal, IAA concentration gradient with high levels of IAA in the root tip appears to control LRP initiation, in contrast to their emergence. An increased ability of the root system to synthetize IAA is observed 10 days

after germination, and this in turn is reflected in the reduced dependence of the lateral root emergence on aerial tissue-derived auxin at this stage (Bhalerao et al., 2002). NPA arrests lateral root development by causing IAA to accumulate in the root apex while reducing levels in basal tissues critical for lateral root initiation. This pattern of IAA redistribution is consistent with NPA blocking basipetal IAA movement from the root tip (Casimiro et al., 2001). These evidences taken together suggest that basipetal and acropetal PAT activities are required during initiation and emergence of lateral roots, respectively.

Analysis of root hair phenotype of auxin- and ethylene-related mutants, such as *aux1*, *axr1*, *axr2*, *axr3*, *ctr1*, *ein2*, *eto1* and *etr1*, implicated both these phytohormones as positive regulators of root hair development (Leyser et al., 1996; Lincoln et al., 1990; Tanimoto et al., 1995; Wilson et al., 1990). Intracellular level of auxin plays an important role in regulating root hair initiation and the ethylene response in *Arabidopsis* root growth (Rahman et al., 2002), suggesting cross talk between these two hormone signaling pathways. The root hair development process seems to be divided into two steps. In the first step, endogenous auxin plays a compensating role in the absence of an ethylene response as observed in *ein2-1* roots, and in the second step, endogenous auxin acts together with ethylene for root hair outgrowth (Pitts et al., 1998).

1.4.3 Regulation of polar auxin transport

Although the mechanisms controlling the polarity and amount of auxin transport during plant growth and development are still unclear, several recent studies have provided insight into regulatory strategy that govern auxin transport. Below I will summarize some of the existing experimental evidences indicating that reversible protein phosphorylation, auxin transport inhibitors, interaction with the actin cytoskeleton and vesicular cycling mechanisms are involved in the modulation of polar auxin transport.

Protein kinases and protein phosphatases catalyze covalent post-translational modifications that result in extremely rapid and reversible changes in protein function in response to changing cellular environmental conditions. In *Arabidopsis* the analysis of *rcn* and *pid* mutants (already mentioned in section 1.3.1) indicates that PP2A phopshatase and PID kinase proteins play a key regulatory role in processes that involve auxin transport or redistribution (Christensen et al., 2000; Rashotte et al., 2001). *rcn1* mutants exhibit reduced PP2A activity in extracts and defects in root curling and gravity response;

both phenotypes are reminiscent of auxin transport defects (Deruere et al., 1999; Garbers et al., 1996). Inhibition of phosphatase activity by treatment with cantharidin (a phosphatase-inhibitor) has been shown to alter activities of the acropetal and basipetal auxin transport in seedlings roots. Both the *rcn1* mutation and phosphatase-inhibitor treatment of wild-type seedlings produce similar effects, implying that PP2A activity is specifically involved in regulating IAA transport. Indeed, basipetal transport is 1.5-fold greater in tips of *rcn1* than in wild-type root tips, and acropetal auxin transport assays reveal that *rcn* mutant is characterized by a dramatic loss of NPA inhibition. The mechanism by which phosphorylation affects auxin transport is unknown. Among different hypotheses, an intriguing one is that phosphorylation might affect transport controlling the biosynthesis of endogenous regulatory compounds such as flavonoids (Brown et al., 2001). One report indicates that protein phosphatase inhibitors prevent the induction of flavonoid biosynthesis (Christie and Jenkins, 1996).

Although the mechanism by which auxin transport inhibitors (ATIs) control auxin efflux is not known, recent evidences suggest that ATIs might act blocking actin-dependent cycling of auxin transporters between the plasma membrane and an internal membrane compartment (Geldner et al., 2001; Gil et al., 2001); as a result of this observation, the IAA efflux complex would then consists of a dynamic, rather than a stable, association between transport proteins and regulatory proteins.

Protein sorting through directed vescicle targeting is crucial for the establishment of protein asymmetry (Nelson and Grindstaff, 1997), whereas attachment to the actin cytoskeleton maintains the asymmetric localization (Colledge and Froehner, 1998). Experimental evidences directly linked vescicle targeting with localization of PIN proteins (Geldner et al., 2001; Steinmann et al., 1999). Plasma membrane localization of PIN1, PIN2 and PIN3 is regulated by rapid actin-dependent endocytotic cycling between the plasma membrane and endomembrane compartments (Friml et al., 2003; Geldner et al., 2001). When treated with brefeldin A (BFA), an ihnibitor of Golgi vescicle secretion, PIN1, PIN2, PIN3 and AUX1 reversibly aggregated in endocytotic juxtanuclear compartments (Abas et al., 2006; Friml et al., 2002b; Grebe et al., 2002). Proper PIN1 localization and function are dependent on the GNOM/EMB30 auxin response factor (ARF) GDP/GTP exchange factor (GEF), which mediates BFA internalization (Geldner et al., 2001; Muday et al., 2003; Steinmann et al., 1999). In fact, PIN1 is mislocalized in embryos of *gnom* mutant, as in BFA-treated roots, suggesting that GNOM might be the protein that mediates BFA inhibition of auxin transport (figure 14).

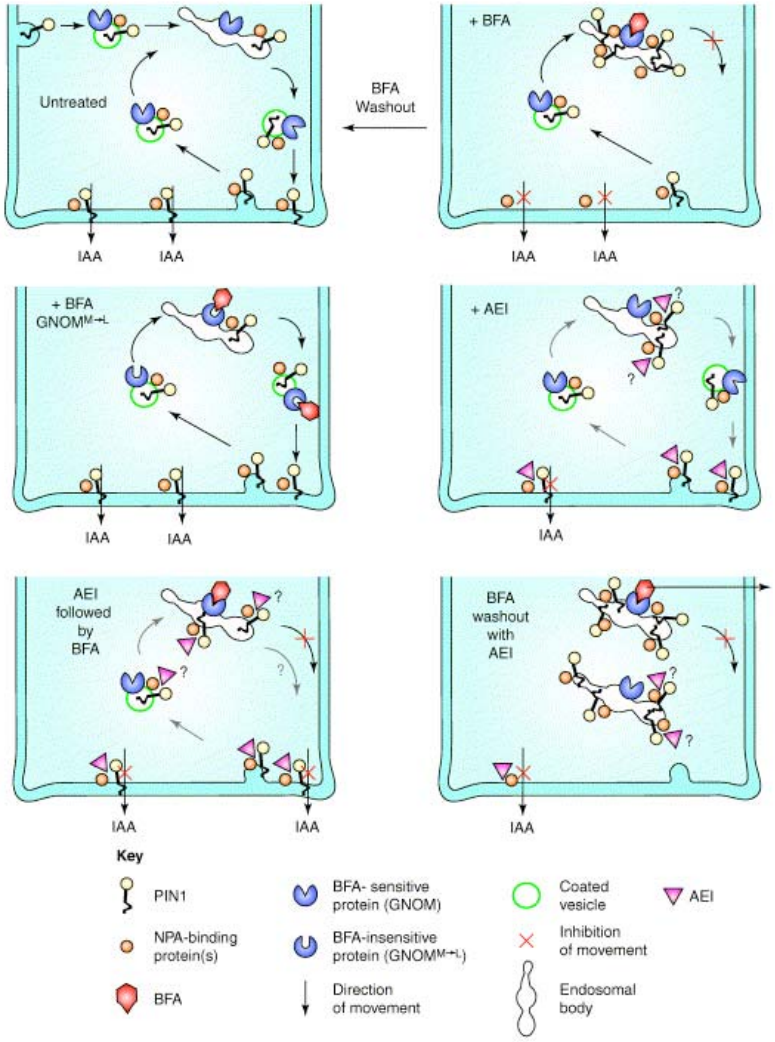


Figure 14. A model of interaction of brefeldin A (BFA) and auxin efflux inhibitors (AEIs) with PIN1 cycling and auxin efflux. From Muday et al., 2003

Several evidences support the hypothesis that an NPA binding protein interacts with actin filaments (Butler et al., 1998; Cox and Muday, 1994; Hu et al., 2000). Moreover, treatment of corn coleoptiles with cytochalasin B (drugs that fragment actin filaments) reduces PAT and the regulation of PAT by NPA (Butler et al., 1998). Cytochalasin D-treated cells show reduced polar localization of PIN1, with some cells exhibiting irregular point of internal PIN1 accumulation. Both the effect of BFA and the relocalization of PIN1 following BFA washout are blocked by cytochalasin D treatment (Geldner et al., 2001). Taken all together, these data indicate that actin filaments might be required for the initial polar localization and for subsequent vesicle-mediated cycling of efflux carriers.

1.4.4 Flavonoids as endogenous regulators of polar auxin transport

Among all the regulatory strategy that I have discussed above, there is one intriguing possibility that naturally occurring small endogenous molecules, such as flavonoids, can modulate the polarity of auxin transport in plant. Indeed flavonoids have characteristics that make them suitable as endogenous regulators of auxin transport. They display a thight relationship between structure and function that allows a narrow subset of chemical modifications, eventually leading to compounds with a diversity of functions (Stafford, 1990). Flavonoids synthesis is tied to environmental and developmental changes, like wounding, pathogens, symbiotic bacteria and gravistimulation, which result in alteration of auxin transport (Sakuta, 2000; Shirley, 1996). In other words, the changes in flavonoid concentration and distribution in response to changes in gravity vector or other environmental factors could regulate auxin transport to allow growth changes in response to differing environmental conditions. Moreover, flavonoids are localized to the tissues that transport auxin (Murphy et al., 2000) and to the plasma membrane where the auxin transport inhibitor binding site is localized (Dixon et al., 1996).

The idea that flavonoids can modulate PAT is actually not that new: it was for the first time proposed in 1970s (Marigo and Boudet, 1977; Stenlid, 1976), when it was shown that plants grown on quinic acid accumulated phenolic compounds, including but not limited to flavonoids, and had reduced auxin transport. Since then, several papers have been published supporting this idea. Jacobs and Rubery demonstrated in 1988 that flavonoids reduce polar auxin transport in zucchini microsomal vesicles and can compete with NPA for its binding site, with quercetin being the most active compound. Bernasconi et al. found that genistein could displace and compete with NPA bindig to membrane vesicles, thus introducing a model of flavonoid modulation of the phosphorylation state of regulatory proteins associated with auxin efflux (Bernasconi, 1996). Some time later, in Professor Murphy's laboratory (Murphy et al., 2000), it was shown that quercetin and kaempferol displace NPA binding from membranes isolated from *Arabidopsis* plants. Moreover, Fischer et al reported that quercetin produces developmental alterations that are similar to those produced by NPA in wheat embryos (Fischer et al., 1997). *In vivo* evidences for flavonoids as auxin transport regulators came from the analysis of *Arabidopsis tt* mutants that, as I already discussed harbour mutations in genes encoding flavonoid biosynthetic enzymes (figure 15). The most striking and convincing phenotype is *tt4*. *tt4* mutants have elevated auxin transport activity in young seedlings, roots or inflorescences, consistent with the absence of an endogenous negative

auxin transport regulator (Brown et al., 2001; Buer and Muday, 2004; Murphy et al., 2000; Peer et al., 2004). Abundance of mRNA-encoding members of the PIN family is altered in *tt* backgrounds, suggesting that flavonoids may regulate synthesis of auxin transport proteins, not just the activity of the existing ones (Lazar and Goodman, 2006; Peer et al., 2004). Consistent with flavonoid abundance affecting transcription is the localization in the nucleus of both flavonoid biosynthetic enzymes and flavonoid products (Saslowsky et al., 2005). However, the precise mechanisms by which flavonoids modulate PAT remain unknown.

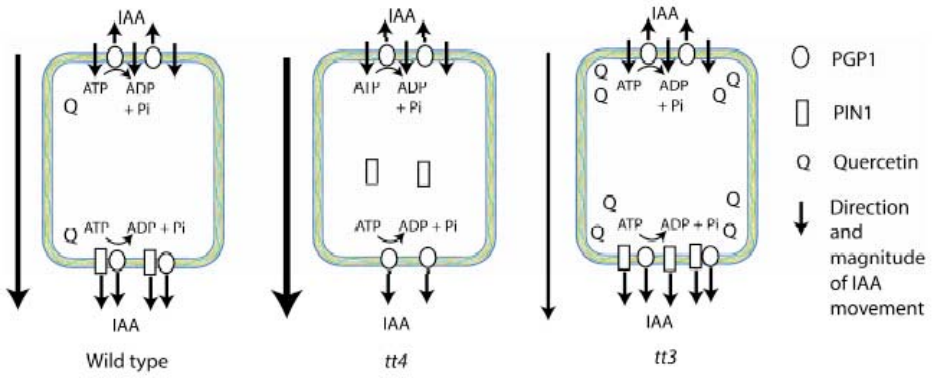


Figure 15. Flavonols and auxin transport. From Peer and Murphy, 2006

1.4.5 Flavonoids and gravitropism

A particular interesting feature of auxin transport polarity is its responsiveness to directional light sources and changes in the perceived gravity vector (Friml et al., 2002b). In addition to polar transport down the axes of plant tissues, auxin can also move laterally accross gravity- or light- stimulated shoots and roots. These environment-induced relocations of auxin create thight coordination of differential cellular growth along longitudinal cell files, allowing growing plant organs to perfom rapid tropic bending in response to changes of environmental cues.

In roots, gravity is perceived mainly in the columella cells at the root cap by sedimentation of starch grains (statoliths), whereas the differential growth response associated with gravistimulation occurs in the elongation zone (EZ) and is a result of lateral auxin transport. Upon gravistimulation, differential basipetal auxin transport intensity between the two sides of the root promotes asymmetric redistribution of auxin to the lower side. Eventually, the consequent local inhibition of elongation growth at the lower side leads the root to bend down in the direction of the gravity vector (figure 16). This accentuated growth on the upper side of gravity-stimulated *Arabidopsis* roots was directly observed (Buer and Muday, 2004) and auxin-responsive reporters have been used successfully to visualize dynamic lateral auxin gradients (Boonsirichai et al., 2003; Luschnig et al., 1998; Ottenschlager et al., 2003; Rashotte et al., 2001).

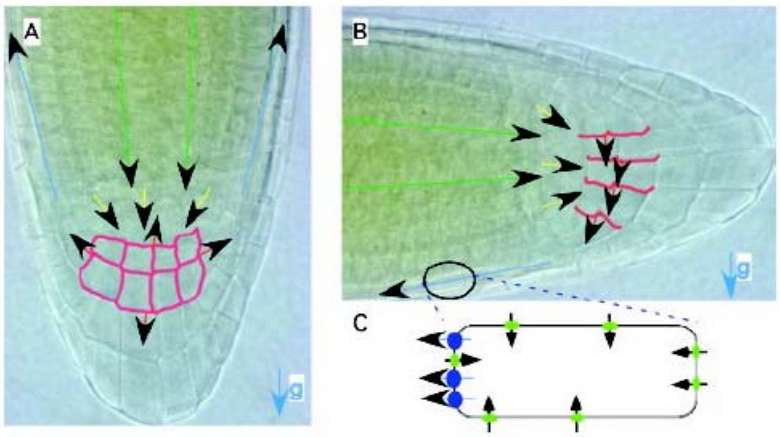


Figure 16. The fountain model of auxin transport in vertically grown roots **(A)** and upon gravity stimulation **(B)**. Polar auxin transport within cell files is mediated by auxin influx and efflux carriers **(C)**. The gravity vector (g) is indicated by a blue arrow in A and B.

Arabidopsis PIN and AUX1 proteins are candidates that may facilitate this polarized auxin transport across gravi-stimulated roots. Gravity-induced retargeting of PIN3 to the lower face of columella cells helps create the initial lateral auxin gradient (Friml et al., 2002b), whereas the expression patterns of the auxin influx and efflux facilitators AUX1 (Swarup et al., 2001) and PIN2 (AGR1/EIR1/WAV6) (Chen et al., 1998; Luschnig et al., 1998; Muller et al., 1998; Utsuno et al., 1998) seem to channel auxin from the root cap to the elongation zone. Very recently, an outstanding work by Swarup et al. provided new insight into the role of AUX1 in root gravitropism (Swarup et al., 2005). Targeted expression of AUX1 demonstrated that root gravitropic response requires auxin to be transported via lateral root cap (LRC) to all elongating epidermal cells and is dependent on AUX1 LRC and epidermal (but not columella) expression domain, presumably to facilitate the delivery of gravity-induced lateral auxin gradient to elongation zone-tissues. At the same time, Abas and co-workers in the laboratory of Christian Lusching demonstrated a critical role for PIN2 in the establishment of the lateral auxin gradients in

EZ of gravity-stimulated roots (Abas et al., 2006). Gravistimulation of wild type roots resulted in asymmetric PIN2 distribution, with more protein degraded at the upper side, consistent with a role of PIN2 in facilitating basipetal auxin transport in the root elongation zone. Post-translational modification, such intracellular trafficking and proteasome activity have been shown to combine to control PIN2 cycling during gravistimulation. Morover, PIN2 localization and degradation were perturbed in a *pin2* (*wav6-52*) allele mutant and this correlates with mutant deficiencies in gravitropic root growth.

Although evidences coming from these two breaktrough-papers helped better understanding of root gravitropism at the molecular level, precise mechanisms involved in the regulation of auxin transport during gravitropic responses are still largely unknown. Among different possible mechanisms, localized synthesis of flavonoids has been shown to modulate the rate of gravity response (Buer and Muday, 2004). A comparison of the root gravitropic response of the wild type and the *tt4* mutant identified a lag in gravitropic curvature in *tt4*. Chemical complementation of *tt4* by naringenin reinstated flavonoid production and restored wild type gravity response, consistent with a role for flavonoids in controlling the flow of auxin needed for root gravitropism. Reorientation of plants relative to the gravity vector leaded to and induction of flavonoid synthesis in the epidermal tissues of *Arabidopsis* root tip, suggesting that flavonoids function to control auxin transport from the tip back to both sides. In conclusion, induction of flavonoid synthesis in response to environmental stimuli seems to alter auxin transport to allow regulation of plant growth and development.

The concept of flavonoids as positive mediators of plant gravitropism fascinated me. Therefore, I tried to address this question in more detail. I investigated the role of flavonoids in root gravitropism by comparing the flavonoid accumulation profiles and gravitropic responses of *pin2* mutants with the wild type. Interesting and surprising results are summarized and discussed in section 4.6.

1.4.6 Flavonoids and cell growth

Cell growth is largely determined by the extension rate of the cell wall, which is a complex structure consisting of cellulose, hemicellulose and pectin, and a number of structural proteins (Carpita and Gibeaut, 1993; Cosgrove, 1993). The major components of the pectic matrix are homogalacturonan (HGA), rhamnogalacturonan I (RG I), and rhamnogalacturonan II (RG II) (Ridley et al., 2001). Sophisticated sugar biosynthetic machinery is required to synthesize the monosaccharides that form the cell wall carbohydrates (Seifert, 2004). Pectins are synthesized in the Golgi apparatus by glycosyltransferases using nucleotide sugars as donor substrates (Scheible and Pauly, 2004). Rhamnose (Rha), a major component of RG I and RG II, has been hypothesized to be synthesized in *Arabidopsis thaliana* by a family of three highly similar Rhamnose Biosynthesis (RHM) proteins that convert UDP-D-Glc to UDP-L-Rha (Reiter and Vanzin, 2001).

During cell wall expansion, many polysaccharides need to be rearranged. This is conducted by a number of specific hydrolytic enzymes, such as the xyloglucans endotransglucosylases/hydrolases that act on xyloglucans and the polygalacturonases that act on pectins, and by other proteins, such as nonhydrolyzing expansins (Cosgrove, 1999). Several different components such as glycosylphosphatidylinositol (GPI)-anchored membrane proteins, wall associated kinases, and the microtubule and actin cytoskeletons have been shown to be important for cell shape determination (Gapper and Dolan, 2006; Ringli et al., 2002; Roudier et al., 2005; Wagner and Kohorn, 2001).

In the laboratory of Dr. Christoph Ringli, at the Institute of Plant Biology in Zürich, a new gene – *LRX1* - involved in the regulation of the cell wall formation has been identified and characterized (Baumberger et al., 2003a; Baumberger et al., 2003b). *LRX1* is a member of a family of 11 genes enconding for extracellular proteins. LRX1 consists of an N-terminal leucine-rich repeat (LRR) domain, involved in protein-protein interaction, and a structural extension moiety at the C terminus, which can modify the properties of the cell wall and might play a role in connecting the cell wall with the plasma membrane by anchoring target proteins (Cassab, 1998; Knox, 1995). *LRX*1 sinergistically interacts with its paralogous gene *LRX2*, and ultrastructural analysis revelead severe deficiencies in the cell wall architecture of *lrx1 lrx2* double mutant, indicating a role for *LRX1* and *LRX2* during cell wall formation (Baumberger et al., 2003b).

Few years later, in order to identify genes that are likely to be involved in the same developmental process as LRX1, a suppressor screen was carried out on the *lrx1* mutant and two independent *rol1* (for *repressor of lrx1*) alleles were identified (Diet el al., 2006). The *ROL1* locus encodes RHM1, which is involved in the formation of UDP-L-Rha. Consistently, the *rol1* mutations cause aberrant RG I production. Because L-Rha is an abundant component of pectic polysaccharides but absent from other cell wall components, these data suggest that LRX1 might be an extracellular regulator involved in the formation of the pectin matrix (Diet et al., 2006).

A more detailed analysis of the *rol1* mutants by Ringli and co-workers revealed hyponastic growth, aberrant pavement cell morphology in cotyledons and defective thricome formation. The fact that the main flavonols of the Arabidopsis shoot are rhamnosylated (Jones et al., 2003; Veit and Pauli, 1999) led to the hypothesis that an alteration in flavonoids accumulation in the *rol1* mutants might occur and have an influence on plant development. In order to investigate this hypothesis, in collaboration with Christoph Ringli, we performed a targeted metabolite profile analysis of *rol1* mutants by HPLC-ESI-MS/MS. The results obtained during this collaboration are summarized and discussed in section 3.7.

2 AIM OF THE WORK

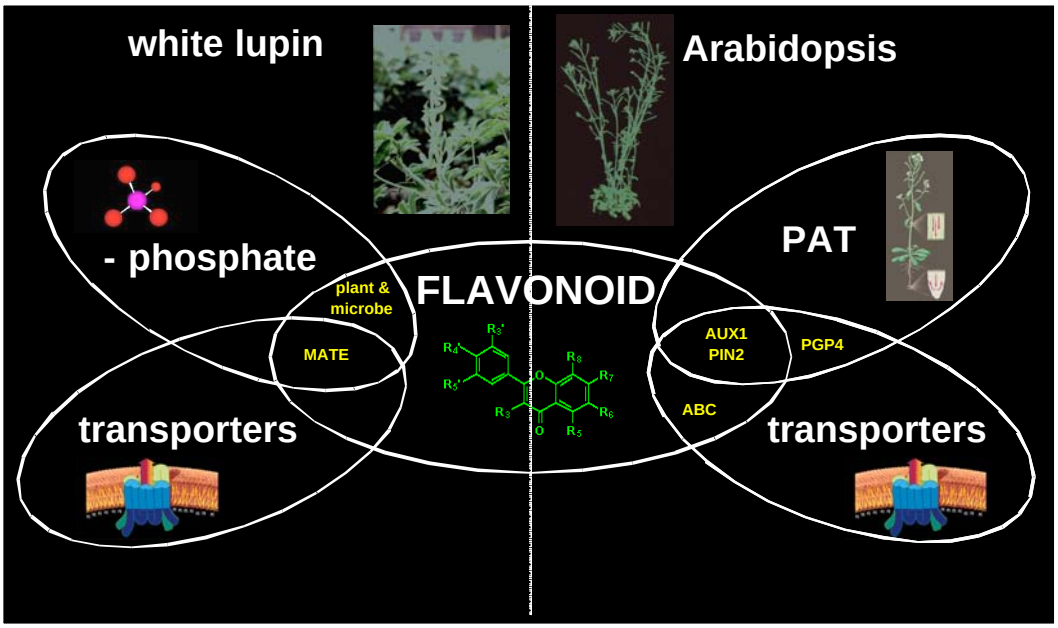
The aims of the work presented here, were to investigate:

(i) The role of root flavonoids as signal molecules in plant-microorganism interactions, using white lupin rhizosphere as a paradigm;

(ii) The transporters involved in the exudation of phytochemicals (mainly flavonoids) from white lupin cluster roots under phosphate starvation, and from *Arabidopsis thaliana* roots;

(iii) The role of flavonoids as agents in plant developmet, especially their regulation of polar auxin transport in plant cells, including the characterization of a new auxin transport protein, AtPGP4.

In the figure below the network of topics exlpored to achieve these general aims is illustrated. Investigations have involved several domains of plant biology: the organic chemistry of root flavonoids; the cellular transport mechanisms of phytochemicals and auxin; the flavonoid cell biology; the microbiology of the rhizosphere and the root developmental processes.



3 RESULTS

Short summaries and reprints of publications describing the results obtained during my PhD work

3.1 Isoflavonoid exudation from white lupin roots is influenced by phosphate supply, root type and cluster-root stage

Laure Weisskopf, Nicola Tomasi, **Diana Santelia**, Enrico Martinoia, Nicolas Bernard Langlade, Raffaele Tabacchi and Eliane Abou-Mansour

Reprinted from: New Phytologist (2006) **171**:657-68

We undertook the characterization of the isoflavonoid profiles produced in and excreted from growing cluster roots, in order to extend our knowledge of the secretion physiology beyond organic acids. We focused on this particular class of secondary compounds, which has been shown to be involved both in nutrient acquisition (nitrogen and iron) and in plant microbe interactions (attraction of nitrogen-fixing bacteria or antimicrobial activity against fungi).

With the help of chemists of the University of Neuchâtel (Dr. E. Abou-Mansour and Prof. R. Tabacchi), we were able to identify the major isoflavonoids present in and secreted from white lupin cluster roots and we investigated the effect of phosphorus supply, root type and cluster root stage on the production and secretion of isoflavonoids. This is to our knowledge the first report on phenolics secreted by cluster roots in any plant species and constitutes an important progress towards a more complete understanding of cluster root secretion physiology.

New Phytologist

Research

Isoflavonoid exudation from white lupin roots is influenced by phosphate supply, root type and cluster-root stage

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Received: *7 February 2006*
Accepted: *2 April 2006*

Summary

• The internal concentration of isoflavonoids in white lupin (*Lupinus albus*) cluster roots and the exudation of isoflavonoids by these roots were investigated with respect to the effects of phosphorus (P) supply, root type and cluster-root developmental stage.

• To identify and quantify the major isoflavonoids exuded by white lupin roots, we used high-pressure liquid chromatography (HPLC) coupled to electrospray ionization (ESI) in mass spectrometry (MS).

• The major exuded isoflavonoids were identified as genistein and hydroxygenistein and their corresponding mono- and diglucoside conjugates. Exudation of isoflavonoids during the incubation period used was higher in P-deficient than in P-sufficient plants and higher in cluster roots than in noncluster roots. The peak of exudation occurred in juvenile and immature cluster roots, while exudation decreased in mature cluster roots.

• Cluster-root exudation activity was characterized by a burst of isoflavonoids at the stage preceding the peak of organic acid exudation. The potential involvement of ATP-citrate lyase in controlling citrate and isoflavonoid exudation is discussed, as well as the possible impact of phenolics in repelling rhizosphere microbial citrate consumers.

Key words: genistein, isoflavonoids, *Lupinus albus* (white lupin), phosphate deficiency, proteoid roots.

New Phytologist (2006) **171:** 657–668

doi: 10.1111/j.1469-8137.2006.01776.x

Introduction

In nature, plants often grow in soils with low available phosphate. To cope with this problem, they have developed several resistance mechanisms (Schachtman *et al.*, 1998; Raghothama, 1999). Two main strategies are the association with mycorrhizal fungi and the formation of particular root structures called 'cluster roots' or 'proteoid roots' (Purnell, 1960). In most cases, plants form either cluster roots or mycorrhizas, but more and more exceptions are being found to this rule of mutual exclusion of the two strategies (Shane & Lambers, 2005a and references therein). Cluster-root formation is not as common in plants as mycorrhizal symbioses, but it is a very efficient strategy to cope with phosphate deficiency and does not depend on the availability of the fungal partner. Cluster roots release large amounts of organic acids into the rhizosphere (Neumann & Martinoia, 2002; Lamont, 2003; Shane & Lambers, 2005a; Le Bayon *et al.*, 2006) and these organic acids solubilize phosphate by chelation and ligand exchange (Dinkelaker *et al.*, 1989, 1997; Gerke *et al.*, 1994, 2000; Jones & Darrah, 1994; Ryan *et al.*, 2001; Dakora & Phillips, 2002). Cluster-root physiology and ecology have been intensively studied over the last decade

(Skene, 1998; Watt & Evans, 1999; Neumann & Martinoia, 2002; Lamont, 2003; Shane & Lambers, 2005a,b) and particular interest has been devoted to the exudation of organic acids. However, not much attention has been paid so far to other exuded molecules such as phenolics, despite their potential role in plant nutrition (Dinkelaker *et al.*, 1995; Marschner, 1995) and plant–microbe interactions (Dakora & Phillips, 1996; Paiva, 2000).

Accumulation of phenolics is a well-known symptom of nutrient stress, and different classes of phenolic compounds are produced depending on the nature of the stress: phosphate deficiency is known to induce anthocyanin accumulation, while the production of phenolics may be up-regulated under iron starvation, and flavonoids, as well as isoflavonoids, have been linked to nitrogen stress (Dixon & Paiva, 1995). Previous reports have studied the impact of phenolics on metal resistance, with resistance to aluminium being investigated in maize (*Zea mays*) (Kidd *et al.*, 2001) and resistance to copper in alfalfa (*Medicago sativa*) (Parry & Edwards, 1994) and white lupin (*Lupinus albus*) (Jung *et al.*, 2003). Jung and coworkers showed that isoflavonoids are able to bind copper ions and speculated that this might reduce copper toxicity. This phenomenon might be of general interest for cluster-rooted species. Exudation of organic anions is often accompanied by rhizosphere acidification. For many heavy metals, solubility increases when pH decreases and phenolics might help to reduce this side effect of phosphate solubilization.

In addition to their role in iron nutrition (Römheld & Marschner, 1983; Zhang *et al.*, 1991; Moran *et al.*, 1997) or heavy metal resistance (Schutzendubel *et al.*, 2001; Jung *et al.*, 2003), phenolics, especially flavonoids, also play a major role in plant–microbe interactions (Paiva, 2000). In legumes, isoflavonoids are an abundant class of phenolic compounds and they have been reported to be involved in many plant–microbe interactions. On the one hand, they can attract mutualistic microorganisms such as nitrogen-fixing bacteria (Dakora *et al.*, 1993) and mycorrhizal fungi (Hirsch & Kapulnik, 1998). On the other hand, many isoflavonoids are involved in the defence response against potential soil-borne bacterial or fungal pathogens. For a review on the various biological roles of isoflavonoids in plant–microbe interactions, see Paiva (2000) or Dakora & Phillips (1996).

Previous studies on isoflavonoids have been conducted in the cluster-rooted species white lupin (*Lupinus albus* L., Fabaceae). However, these studies either focused on the precise profiling and structural elucidation of the various isoflavonoids in white lupins grown in phosphorus (P)-sufficient conditions, and thus without cluster roots (Wojtaszek *et al.*, 1993; Wojtaszek & Stobiecki, 1997; Katagiri *et al.*, 2000, 2001; Sakasai *et al.*, 2000; Bednarek *et al.*, 2001, 2003; Pislewska *et al.*, 2002; Jung *et al.*, 2003), or took phenolics into account only as a general class of compounds in cases where P-deficient white lupins forming cluster roots were studied (Dinkelaker *et al.*, 1995; Neumann *et al.*, 2000). To date, no study has investigated the isoflavonoids produced and exuded by the cluster roots of white lupin plants.

In white lupin, cluster roots can be separated into four different growth stages (Massonneau *et al.*, 2001): the juvenile, the immature, the mature and the senescent stages. These stages differ in the quantity and quality of organic acid exudation: at the juvenile stage, cluster roots are still growing and secrete mainly malate. After 2–3 d, the cluster root reaches the immature stage, where the full size of the root is achieved but no or little exudation of organic acids occurs. High rates of exudation of organic acids, mainly citrate, occur at the mature stage. At this point, a concomitant release of protons causes rhizosphere acidification. However, acidification and carboxylate exudation are two separate processes. Carboxylates are exuded in the unprotonated form through recently identified channels (Kollmeier *et al.*, 2001; Sasaki *et al.*, 2004; Zhang *et al.*, 2004), whereas acidification is a result of the activation of the plasma membrane proton pump (Yan *et al.*, 2002). In addition to protons, sodium and potassium ions have been shown to serve as counterions for citrate release and may be quantitatively more important than protons for the maintenance of charge balance (Zhu *et al.*, 2005). At the senescent stage, almost no carboxylates are exuded.

In the present work, we aimed to extend our knowledge of the physiology of cluster-root exudation beyond the carboxylates to secondary compounds, and we chose to focus our attention on isoflavonoids because of their abundance in legumes and their potential role both in nutrition and in plant–microbe interactions. We took advantage of the development of high-pressure liquid chromatography (HPLC) coupled to electrospray ionization (ESI) in mass spectrometry (MS) to facilitate the identification of conjugated isoflavonoids in cluster roots without the necessity to separate all the compounds. We used this analytical tool to address the following biological questions. (i) Does phosphate status induce changes in the quantity and quality of the isoflavonoids produced and exuded? (ii) Is the isoflavonoid composition different in cluster and noncluster roots? (iii) Is the exudation pattern for isoflavonoids similar to that for organic acids in white lupin cluster roots?

Materials and Methods

Chemicals

The methanol used for liquid extraction was distilled before use. HPLC-grade acetonitrile was purchased from SDS (Peypin, France). β-glucosidase and genistein were purchased from Sigma (Buchs, Switzerland).

Plant material and harvest of different root parts

White lupin plants (*Lupinus albus* L. cv. Amiga; Südwestdeutsche Saatzucht, Rastatt, Germany) were grown either in the presence (+P) or the absence (–P) of a P source, as previously described

by Massonneau *et al.* (2001). Plants were grown at 22°C and 65% relative humidity with a light period of 16 h at 200 μmol m^{-2} s^{-1}. For the separation between cluster and noncluster roots (without differentiation of the cluster-root stages), cluster roots of all developmental stages were harvested and pooled, while noncluster roots consisted of entire secondary roots without cluster roots. The different stages of cluster roots were harvested as described by Massonneau *et al.* (2001). In order to differentiate between the developmental stages of root clusters, the root system was immersed in a pH-indicator solution, which indicates acidification in mature cluster roots (Neumann *et al.*, 1999).

Extraction of phenolic compounds and HPLC analysis

Excised root parts were washed in distilled water and subsequently incubated in 4 ml of water for 1 h at room temperature under gentle shaking to allow the exudation of root exudates (modified from Neumann *et al.*, 2000). The root exudates were collected and frozen at −80°C. After freeze-drying, 2.5 ml of 80% methanol was added in four steps (1 ml initially, followed by three additions of 0.5 ml). Each step was followed by vigorous shaking and filtration at 0.45 μm (Schleicher & Schuell, Dassel, Germany). The remaining roots were then incubated in 4 ml of 80% methanol [volume/volume (v/v)] for 1 h at room temperature under gentle shaking to recover the internal cell concentrations. Internal concentration extracts were filtered at 0.45 μm (Schleicher & Schuell). After solvent evaporation, extracts were resuspended in the first HPLC solvent (A) in proportion to the root fresh weight (FW) (0.75 μl mg^{-1} root FW for the exudation samples and 1.5 μl mg^{-1} root FW for the internal samples). Volumes of 50 μl were loaded on a Machery-Nagel (Düren, Germany) Nucleosil 100-5 C18 column (254 × 4 mm) protected by a precolumn of the same material. Gradient elution was performed by varying the proportion of solvent A [acetonitrile (MeCN):H_2O:acetic acid (AcAc), 5 : 93 : 2, v/v/v] and solvent B (MeCN:H_2O:AcAc, 75 : 23 : 2, v/v/v). Starting at 10% of solvent B, the proportion was programmed to reach 52% in 16 min, 75% in 22 min and 100% in 25 min, and solvent B was maintained at 100% for another 5 min. The total analysis time was 40 min including column wash and stabilization. The flow rate was set to 0.4 ml min^{-1} and detection to 254, 264 and 320 nm. For quantification of the exuded amounts of genistein, genistein 7-*O*-diglucoside, genistein 6″-*O*-malonyl-diglucoside and genistein 6″-*O*-malonyl-O-glucoside, calibration curves were elaborated with isolated compounds from cluster-root exudates. Single compounds were obtained by separately collecting peaks after HPLC analysis. The peak purity was assessed by HPLC after collection of individual compounds and calibration curves were obtained based on the peak area (absorption at 263 nm). All analyses were performed with three or four replicates, each replicate representing the harvest of about five boxes containing 12 plants each.

Structural elucidation: LC-ESI-MS analysis

In leguminous plants, a small part of isoflavonoid glucosides is present as acylated conjugates (Barnes *et al.*, 1994). LC-ESI-MS has been previously used for the detection of isoflavonoid glycosides and free aglycones in extracts of white lupin roots (Stobiecki *et al.*, 1999; Bednarek *et al.*, 2001; March *et al.*, 2004; Kachlicki *et al.*, 2005). On the basis of mass to charge ratio (m/z) values of protonated $[M+H]^+$ and fragment ions, it is possible to identify an acyl substituent linked to a sugar moiety in an isoflavonoid glycoside.

HPLC analysis was performed in an acetonitrile gradient in water using acetic acid as a modifier. Acidification of the mobile phase not only improved resolution for liquid chromatography but also played an important role in the ionization process by decreasing the relative contribution of the $[M+Na]^+$ ions in the MS, as previously observed by Stobiecki *et al.* (1999). A Hewlett Packard 1100 HPLC system (Hewlett Packard, Palo Alto, CA, USA) equipped with a binary pump was used with a photodiode array spectrophotometric detector and coupled to an Agilent 1100 series LC/MSD Trap instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with an electrospray ionization source, all controlled by the Agilent CHEMSTATION software. Separation was performed as mentioned above. For positive and negative ionization mode in ESI-MS analysis, the pressure of the nebulizer (nitrogen) was set at 50 psi (corresponding to 3.44 bar) dry gas flow at 7 ml min^{-1} and the temperature of the drying gas (N_2) at 300°C. The capillary voltage was set at 3660 V in positive and 2900 V in negative ionization mode. The voltage of the skimmer lens and the entrance lens in the ion source were automatically optimized by direct inlet of a solution of genistein, hydroxygenistein and genistein glucoside (at a concentration of 1 μg ml^{-1} in MeCN:H_2O:AcAc; 1 : 1 : 0.02). Hydroxygenistein and genistein-7-*O*-glucoside were isolated from white lupin leaf extracts and structural identification was based on nuclear magnetic resonance and MS data according to the literature (Ingham, 1976; Ingham *et al.*, 1983; Tahara *et al.*, 1984; Murthy *et al.*, 1986).

The relative intensities of the molecular species and fragment ions were dependent on the ionization mode. Ion chromatograms resulting from LC-ESI-MS analyses of the root exudates extract are shown in Fig. 1, together with LC-UV traces obtained in parallel. In the positive ion mode analysis (Supplementary Fig. S1a), the main peak $[M+H]^+$ was observed and no fragmentation occurred. By contrast, in the negative ion mass spectra (Supplementary Fig. S1b), Y_n-type fragment ions were mainly observed corresponding to the genistein moiety Y_0^- m/z 269 or the hydroxygenistein moiety Y_0^- m/z 285. The elimination of 44 atomic mass units (u) was connected with the elimination of an acetyl group leading to the fragment ion m/z 473, and the elimination of a $COCH_2COO$ fragment (86 u) derived from malonate, giving rise to an ion at m/z 431 (genistin) and 447 (hydroxygenistin), while cleavage of the

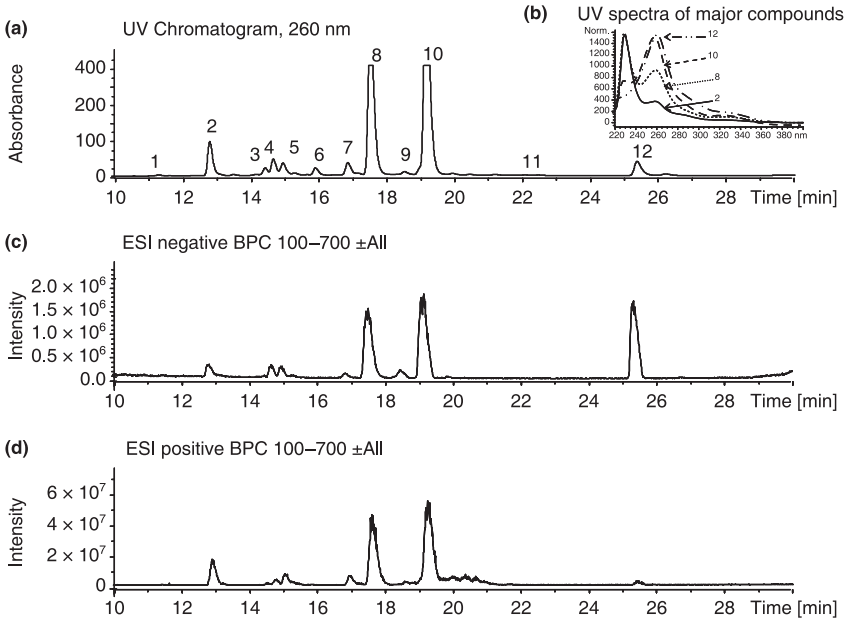

Fig. 1 Liquid chromatography (LC) and ultraviolet (UV) chemical profiles of white lupin (*Lupinus albus*) root extracts. (a) Liquid chromatography–ultraviolet (LC-UV) chromatogram; (b) UV spectra of major compounds; (c) liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) $[M-H]^-$ ion and (d) LC-ESI-MS $[M+H]^+$ ion base peak chromatograms (BPCs) of white lupin root exudate extract.

glucosidic bond by loss of 162 u [(glucose (Glc)] gave rise to the aglycone moiety ion at m/z 269 (genistein) and 285 (hydrogenistein). The abundance of the fragments [M-H-Mal]$^-$ and [M-H-Mal-Glc]$^-$ (where Mal is malonyl) was very important compared with [M-H]$^-$, giving rise to an ion corresponding to the aglycone.

It was not possible to establish unambiguously the position of the sugar substitutions on genistein diglucoside, and two possibilities are suggested: the compound could be genistein 7-*O*-diglucoside or a structure deduced from literature data, which indicate that glycosylation might occur on the 4′- and 7-hydroxyl group of genistein, such a structure having been identified in hairy root cultures of lupins (Berlin *et al.*, 1991). The malonyl group is probably in the 6′-position of the sugar part, and this hypothesis is supported by the fact that to date only 6′-malonates of the isoflavonoid glucosides have been reported from white lupin. ESI mass spectra and fragmentation diagnostics are presented in supplementary material (Supplementary Figs S1 and S2).

Hydrolysis

Two milligrams of methanolic extract from the roots of white lupin was added to 6 mg of β-glucosidase in 4 ml of acetate buffer, pH 5, at 37°C for 12 h. The sample was then extracted with ethyl acetate, evaporated and redissolved in 200 µl of solvent A. A volume of 10 µl was loaded on the HPLC column for analysis.

Statistical analyses

Analyses of variance were performed with S-PLUS 6 Statistical Software (Insightful Corporation, Seattle, WA, USA) with a confidence interval of 95%. We used one-way analysis of variance (ANOVA) to test the general influence of P status or cluster-root stage for statistical relevance and Student's *t*-test for pairwise comparisons.

Results

Profiling of isoflavonoids in roots and root exudates

The enzymatic hydrolysis of a root exudate extract with β-glucosidase yielded only the free aglycones genistein and hydroxygenistein, establishing the β configuration of the glucosidic linkages between aglycones and sugar moieties and the absence of prenylated isoflavonoids.

The isoflavonoids were detected on the basis of their UV absorption at 263 and 350 nm (Fig. 1). Twelve peaks of isoflavonoids (Table 1), identified previously in white or yellow lupin (*Lupinus luteus*) (Shibuya *et al.*, 1991; Franski *et al.*, 1999), were tentatively recognized based on UV spectra, m/z values of protonated molecules $[M+H]^+$ and fragment ions Y_0^- created after cleavage of the glycosidic bonds between sugars and aglycones. In addition to the free aglycones genistein (peak 12) and hydroxygenistein (peak 11), the root exudate extracts contained mono (peaks 6, 8, 9) and diglucosyl (peaks 1, 2) conjugates, as well as mono (peaks 7, 10) and diglucosyl malonyl (peaks 3, 4, 5) conjugates. Genistein conjugates were more abundant than hydroxygenistein conjugates.

Isoflavonoid concentrations and exudation are influenced by P supply

To assess the influence of phosphate supply on the concentrations and exudation of isoflavonoids, phenolics were extracted

Table 1 Isoflavonoids and their glucosides identified in white lupin (*Lupinus albus*) root exudates

Peak number	Compound	$[M+H]^+$
1	Hydroxygenistein 7-*O*-diglucoside	611
2	Genistein 7-*O*-diglucoside	595
3	Hydroxygenistein 6′-*O*-malonyl-glucoside	697
4	Genistein 6′-*O*-malonyl-diglucoside	681
5	Genistein 6′-*O*-malonyl-glucoside 4-*O*-glucoside	681
6	Hydroxygenistein 7-*O*-glucoside	449
7	Hydroxygenistein 6′-*O*-malonyl-glycoside	535
8	Genistein 7-*O*-glucoside	433
9	Genistein 4-*O*-glucoside	433
10	Genistein 6′-*O*-malonyl-O-glucoside	519
11	Hydroxygenistein	287
12	Genistein	271

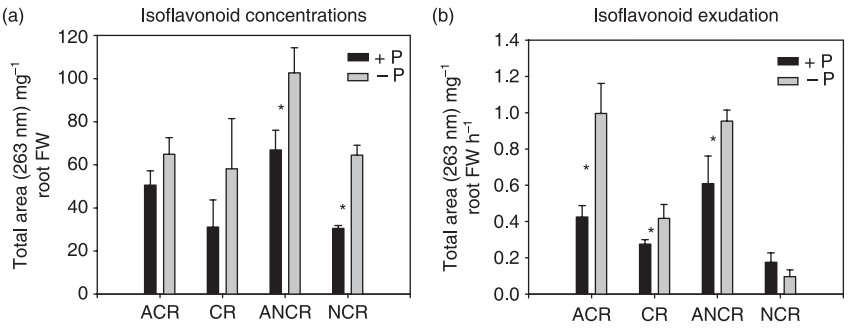

Fig. 2 Effect of phosphorus (P) supply on isoflavonoid concentrations (a) and exudation (b). White lupins (*Lupinus albus*) were grown under P-sufficient (black bars) or P-deficient (grey bars) conditions. Roots were separated into the apex of cluster roots (ACR), cluster roots (without the apex) (CR), the apex of noncluster roots (ANCR) and noncluster roots (without the apex) (NCR). Bars represent means of three replicates. *, significant differences (Student's *t*-test, $P < 0.05$). FW, fresh weight.

from white lupin plants grown either in P-deficient or P-sufficient conditions. As previously observed (Shen *et al.*, 2003), P-sufficient lupin plants also produced cluster roots, but in a much lesser abundance than P-deficient plants. Roots were separated into four fractions: (i) the apex of cluster roots, (ii) cluster roots (without the apex), (iii) the apex of noncluster roots and (iv) noncluster roots (without the apex). Isoflavonoid concentrations and exudation were analysed in the four root types. The total amounts of isoflavonoids were calculated as the sum of the areas of the major peaks in the HPLC profiles detected by UV. When grown under P deficiency, plants produced and exuded significantly more isoflavonoids (ANOVA, $P < 0.05$) than when they were supplied with phosphate (Fig. 2). For isoflavonoid concentrations (Fig. 2a), this effect of P nutrition was especially marked for noncluster roots and their apices (Student's *t*-test, $P < 0.05$), while in cluster roots and their apices no significant difference was observed between plants grown with and without phosphate. As observed for the concentrations, the exudation of isoflavonoids within the same incubation period was generally higher in plants grown in the absence of phosphate (Fig. 2b), except for noncluster roots, where no significant difference was observed.

Phosphate status did not affect all isoflavonoids to the same extent: for example, in cluster roots, a significant increase in genistein (peak 12) concentrations was observed in P-deficient plants (Student's *t*-test, $P < 0.05$). These plants contained 2.3 mg genistein g^{-1} root FW, while only 0.9 mg g^{-1} root FW was detected for plants grown in P-sufficient conditions. In contrast, no effect of P treatment was observed for genistein 6′-*O*-malonyl-O-glucoside (peak 10), which was present at a concentration of approximately 0.2 mg g^{-1} in cluster roots of both P-deficient and P-sufficient plants. Overall, the ratio of internal concentrations vs exudation was not altered by the P treatment.

Isoflavonoid concentrations and exudation are influenced by root type

In order to investigate the influence of root type on isoflavonoids in white lupin, we compared the isoflavonoid concentrations and exudation of cluster and noncluster roots (Fig. 3). For the internal concentrations of isoflavonoids (Fig. 3a,b), higher levels of genistein (peak 12) were found in cluster roots ($P < 0.05$) than in noncluster roots, but no significant difference was observed for the other compounds. In contrast, the exudation of isoflavonoids was generally higher for cluster roots than for noncluster roots (Fig. 3c,d).

Isoflavonoid concentrations and exudation are influenced by cluster-root stage

To assess whether the developmental stage of cluster roots plays a role in isoflavonoid storage and exudation, cluster roots were separated into juvenile, immature, mature and senescent cluster roots. For all root stages, internal isoflavonoid concentrations

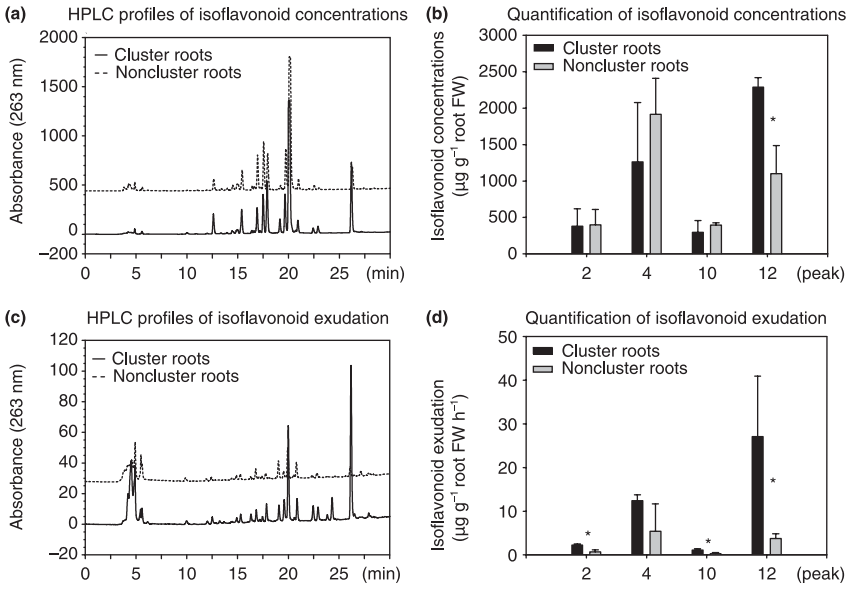

Fig. 3 Effect of root type on isoflavonoid concentrations (a, b) and exudation (c, d). White lupins (*Lupinus albus*) were grown under phosphorus (P)-deficient conditions. Roots were separated into cluster roots and noncluster roots. (a, c) High-performance liquid chromatography (HPLC) profiles of cluster roots (solid line) and noncluster roots (dashed line), for concentrations (a) and exudation (c). (b, d) Quantification of four major isoflavonoids present in and exuded from cluster roots (black bars) and noncluster roots (grey bars). peak 2, genistein 7-*O*-diglucoside; peak 4, genistein 6′-*O*-malonyl-diglucoside; peak 10, genistein 6′-*O*-malonyl-O-glucoside; peak 12, genistein. Bars represent means of three replicates; *, significant differences (Student's *t*-test, $P < 0.05$).

and exudation were determined. The HPLC profiles shown in Fig. 4 are representative examples of the four replicates we analysed for each stage. Only small changes were observed in the concentrations of isoflavonoids (Fig. 4a), but greater differences were observed in isoflavonoid exudation (Fig. 4b) along the growing cluster roots. In contrast with the pattern of organic acid exudation, which is highest in mature cluster roots, most of the isoflavonoid exudation occurred at the beginning of cluster-root development, especially in juvenile and immature cluster roots (Fig. 4b). At the mature stage, exudation decreased and was further reduced at the senescent stage.

In order to obtain a more quantitative idea of isoflavonoid concentrations and exudation, we determined the amounts of the 12 major compounds present in and exuded from the four stages of cluster roots. Overall, isoflavonoid concentrations and exudation varied significantly depending on cluster-root stage (ANOVA, $P < 0.05$). Among the 12 major compounds analysed, six showed an altered pattern in concentration and six in exudation level. Figure 5 shows the pattern for four of these compounds. Although, in the chromatogram (Fig. 4), genistein 6″-*O*-malonyl-O-glucoside (peak 10; retention time (RT) = 21) displayed higher absorbance than genistein (peak 12; RT = 27.5), the quantification based on calibration curves of purified compounds showed that genistein was the compound produced and exuded in the greatest amounts (Fig. 5a). Genistein (Fig. 5a) did not show any significant change in the pattern of exudation or in internal concentrations. For genistein 7-*O*-diglucoside (Fig. 5b), internal concentrations and exudation showed the same pattern: starting high at the juvenile and immature stages, and then decreasing significantly at the mature stage and further diminishing at the senescent stage. While no significant difference was observed for genistein 6′-*O*-malonyl-diglucoside in internal concentrations (Fig. 5c), exudation was higher for juvenile and immature ($P < 0.05$) than for mature and senescent cluster roots. In contrast to genistein 7-*O*-diglucoside and genistein 6′-*O*-malonyl-diglucoside, genistein 6′-*O*-malonyl-O-glucoside (Fig. 5d) showed no difference in concentration between the immature and the mature stages, whereas there was a significant decrease in exudation between these two stages. For these four major compounds, isoflavonoid exudation was higher at the juvenile and immature stages, and then decreased at the mature and senescent stages. We calculated the ratios of exudation vs internal concentrations (Fig. 5e) in order to determine whether or not this decrease in isoflavonoid exudation was a consequence of decreased production. In general, ratios varied significantly with cluster-root stage and genistein was exuded in greater amounts than the other three compounds (ANOVA, $P < 0.05$). For all peaks, the ratio was higher (more exudation) in juvenile and immature cluster roots and lower at the mature and senescent stages, suggesting that the decrease in exudation cannot be explained solely by the decrease in internal concentration. This decrease at the transition from the immature to the mature stage was significant (Student's *t*-test, $P < 0.05$) for genistein 6′-*O*-malonyl-O-glucoside (peak 10). No changes were observed between the mature and the senescent stages.

Discussion

In order to obtain new insights into the exudation physiology of cluster roots, we applied a LC-MS approach to characterize the pattern of isoflavonoids produced and exuded at different stages of white lupin cluster roots. We investigated the effects of phosphate supply, root type and cluster-root stage on the quantity and quality of isoflavonoid concentrations and exudation.

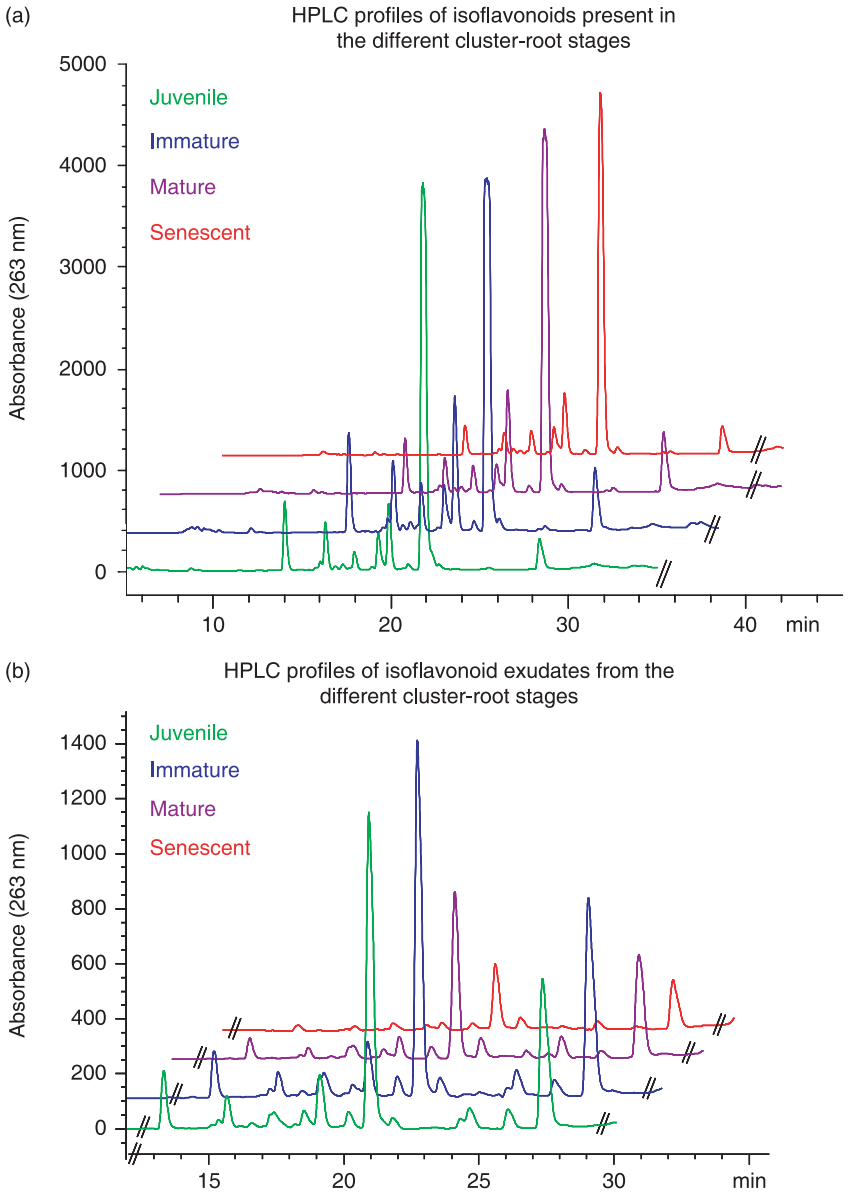

Fig. 4 Effect of cluster-root stage on isoflavonoid concentrations (a) and exudation (b). White lupins (*Lupinus albus*) were grown under phosphorus (P)-deficient conditions. After immersion in a pH indicator solution (see Materials and Methods for more details), cluster roots were separated into four developmental stages: juvenile (green), immature (blue), mature (violet) and senescent (red). Root tissues (concentrations, a) and exudates (exudation, b) were extracted with 80% methanol. Samples were separated on a C18 high-performance liquid chromatography (HPLC) column and absorbance was measured at 263 nm. For internal concentrations (a), 50 µl was injected, corresponding to 35 mg of roots [fresh weight (FW)]. For exudation (b), 50 µl was injected, corresponding to a 1-h exudation of 70 mg of roots (FW).

Major isoflavonoids found in white lupin cluster roots

LC-UV-MS techniques were used for structural elucidation and profiling of flavonoid glycosides in root exudates from white lupin. Four diglycosides, six monoglycosides and two aglycones were identified in the extracts (Fig. 1, Table 1). All recognized compounds have been reported previously in white lupin or other lupin species (Shibuya *et al.*, 1991). Hydrolysis of root extracts with β-glucosidase confirmed the presence of only two aglycones, genistein and hydroxygenistein, and the absence of prenylated compounds. This absence of prenylated compounds was surprising for us, because earlier reports suggested that prenylated isoflavonoids were present in white lupin roots: Tahara *et al.* (1984, 1989) isolated two prenylated isoflavonoids (the monoprenylated lupinalbigenin and the diprenylated 2′-hydroxyisolupinalbigenin) from white lupin roots and Bednarek *et al.* (2001) reported the presence of two monoprenylated isoflavonoids, wighteone and luteone. As the plant growth conditions, the extraction method and the profiling analysis were similar in the cited studies and the present work, a possible reason for the differences in the pattern of isoflavonoids recovered might be the fact that different lupin cultivars were used (*L. albus* cv. Bac by Bednarek *et al.* and *L. albus* cv. Kievskij Mutant by Tahara *et al.* and Katagiri *et al.*, whereas *L. albus* cv. Amiga was used in the present study). In this study, two groups of isoflavonoid conjugates were detected on the basis of the m/z Y_0^- ions, and the major compounds were related to genistein, while the hydoxygenistein conjugates were less abundant.

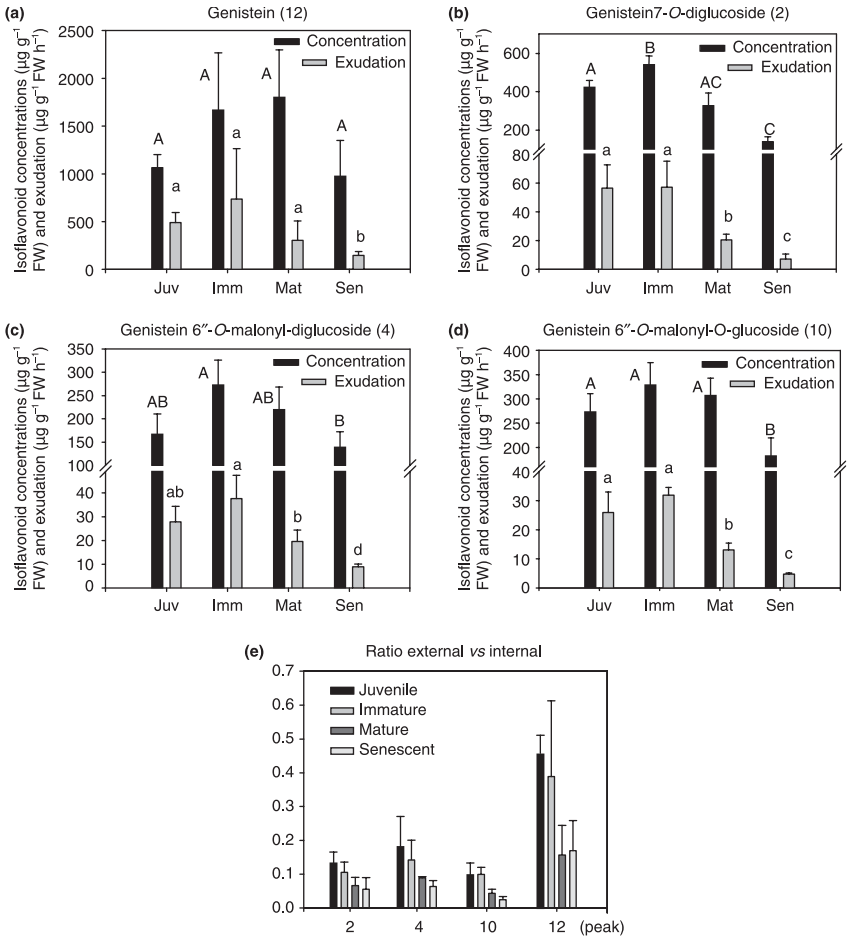

Fig. 5 Quantification (a–d) and external vs internal ratio (e) of four major isoflavonoids in roots and exudates of white lupin (*Lupinus albus*) as a function of cluster-root stage. (a–d) Black bars show the internal concentrations and grey bars show exudation for (a) genistein 7-*O*-diglucoside, (b) genistein 6′-*O*-malonyl-diglucoside, (c) genistein 6′-*O*-malonyl-*O*-glucoside and (d) genistein. Juv, juvenile; Imm, immature; Mat, mature; Sen, senescent. Bars represent means of three replicates. Different letters (uppercase, internal concentrations; lowercase, exuded amounts) indicate statistically different values (Student's *t*-test, $P < 0.05$). (e) Ratio of exudation vs internal concentrations of four major isoflavonoids as a function of cluster-root stage. Cluster roots were separated into four developmental stages: juvenile (black), immature (grey), mature (dark grey) and senescent (white). peak 2, genistein 7-*O*-diglucoside; peak 4, genistein 6′-*O*-malonyl-diglucoside; peak 10, genistein 6′-*O*-malonyl-*O*-glucoside; peak 12, genistein. Bars represent means of three replicates.

Effect of P supply

As a lack of phosphate induces the formation of cluster roots in white lupin, we aimed to assess if the pattern of isoflavonoids would differ in plants grown in P-deficient vs P-sufficient conditions. We found that phosphate deficiency caused a general increase in the concentrations and exudation of isoflavonoids (Fig. 2). Although anthocyanin accumulation is a well-known symptom of phosphate deficiency, the effect of phosphate supply on the production and exudation of isoflavonoids at the root level has not often been investigated. To our knowledge, only three studies reported enhanced phenolic production in plants subjected to phosphate deficiency: Murali & Teramura (1985) in soybean plants (*Glycine max*), Juszczuk *et al.* (2004) in bean (*Phaseolus vulgaris*) and Akiyama *et al.* (2002) in melon (*Cucumis melo*). Juszczuk *et al.* (2004) found that, in bean, phosphate deficiency increased the activity of L-phenylalanine ammonia lyase, and also root exudation of phenolics. However, the chemical nature of the exuded phenolic compounds was not investigated. In the case of nitrogen deficiency, the role of isoflavonoids in the signalling leading to symbiosis with nitrogen-fixing bacteria is well documented and one cannot exclude the possibility that similar compounds might be involved in other kinds of nutrient-deficiency signalling pathways. Supporting this hypothesis, Akiyama *et al.* (2002) found that, in melon roots, P deficiency induced the exudation of a glycosylflavonoid, which is involved in the regulation of the association with arbuscular mycorrhizal fungi. This may indicate that flavonoids, and potentially also isoflavonoids, might be involved in P-deficiency signalling as well as in nitrogen-deficiency signalling.

Differences between cluster and noncluster roots

In comparing the HPLC profiles of cluster and noncluster roots grown in P-deficient conditions (Fig. 3a,c), we found that the profiles were very similar and only differed in the amounts of some isoflavonoids, such as genistein, which was more abundant in cluster roots. Quantitative differences between cluster and noncluster roots were more pronounced for exudation than for internal concentrations, and this suggests that both types of roots are producing the same pattern of phenolic compounds, but that cluster roots are

exudating higher amounts. This result confirms the observation of Neumann *et al.* (2000) that more phenolic compounds were exuded from cluster roots than from noncluster roots, and provides additional information concerning the chemical nature of these phenolic compounds. It thus seems that isoflavonoids, as well as organic acids, are exuded at higher rates by cluster roots than by noncluster roots. Moreover, the results are presented here on a fresh weight basis, and as cluster roots are much heavier than noncluster roots, the exudation of isoflavonoids by a comparable root length segment will be far greater for cluster roots than for noncluster roots.

Pattern of isoflavonoid exudation along growing cluster roots

Thanks to detailed knowledge of the organic acid exudation physiology of cluster roots, we were able to separate growing cluster roots into well-defined developmental stages (Massonneau *et al.*, 2001). It proved useful to analyse the exuded fractions and not only the root concentrations, where no significant difference between stages was found except for the senescent stage, which was characterized by a decreased level of internal isoflavonoids. While most of the organic acid exudation occurs at the mature stage, we showed in this work that the main burst of isoflavonoid exudation takes place before this stage, starting at the juvenile stage and remaining high until the immature stage. The fact that this isoflavonoid burst occurs immediately before the exudation of citrate is of particular interest when considering the potential antimicrobial role of isoflavonoids (Dakora & Phillips, 1996): it could be interpreted as a means for the plant to locally and temporarily reduce microbial density in the rhizosphere of cluster roots, just before the exudation of citrate, and thus to decrease the microbial degradation of this phosphate-chelating agent. We have shown in previous studies (Weisskopf *et al.*, 2005, 2006) that the abundance of the bacterial populations in the rhizosphere of cluster roots is indeed reduced at the stage at which citrate exudation takes place. This protection against microbial citrate degradation was related to several mechanisms, including rhizosphere acidification and production of extracellular chitinases and glucanases, as well as exudation of isoflavonoids.

Interestingly, the pattern of isoflavonoid exudation along growing cluster roots, starting high at the juvenile and immature stages and decreasing at the mature stage, can be related to the expression and activity of a particular enzyme, ATP-citrate lyase (ACL). As previously reported (Langlade *et al.*, 2002), ACL activity is highest at the beginning of cluster-root development and decreases at the mature stage. ACL catalyses the ATP-dependent breakdown of citrate into oxaloacetate and acetyl-CoA. As suggested by Langlade *et al.* (2002), this enzyme could be responsible for the switch in the organic acid preferentially exuded from juvenile to mature cluster roots, by reducing citrate concentrations and providing the malate precursor oxaloacetate. Moreover, ACL activity also produces acetyl-CoA, in addition to oxaloacetate. This acetyl-CoA is used for fatty acid as well as for flavonoid synthesis. Thus, the down-regulation of a single enzyme could explain two important changes in the exudation physiology of growing cluster roots: both the switch from malate to citrate at the mature stage and the decrease in isoflavonoids from the immature to the mature stage.

By comparing the ratios of exudation vs internal concentrations (Fig. 5e), one can observe that genistein is exuded at a higher ratio than the other compounds. Genistein is the most lipophilic molecule among our identified isoflavonoids, and this may account for its ready diffusion across the plasma membrane and for its exudation in greater amounts. In all four compounds analysed, there was a tendency for the external vs internal ratio to be higher for the juvenile and immature stages, compared with the mature and senescent stages. Moreover, because, in addition to genistein, hydrophilic glycosylated genistein conjugates are also exuded, it can be speculated that plasma membrane-localized transporters are involved in the export of isoflavonoids. However, this transport mechanism remains to be elucidated. For the four isoflavonoids quantified, the internal concentrations of immature and mature cluster roots were not significantly different (Fig. 5). However, exudation was significantly reduced at the mature stage in all cases except for genistein. This hints at a possible storage mechanism of these nonexuded compounds in the root cells, probably in the vacuole. We are currently investigating the possible involvement of a multidrug and toxin extrusion transporter, which is highly expressed in mature cluster roots, in isoflavonoid transport. The recent breakthrough by Uhde-Stone *et al.* (2005), who succeeded in transforming white lupin, will have a great impact on future work, enabling more detailed study of the exudation of isoflavonoids and their role in cluster-root metabolism.

In conclusion, we have shown that white lupin roots exuded more isoflavonoids – especially more genistein – when the plants were grown in the absence of phosphate. Cluster roots and noncluster roots showed the same pattern of exuded isoflavonoids, but larger amounts were exuded from cluster roots than from noncluster roots, suggesting that the up-regulated exudation physiology of cluster roots is not restricted to carboxylates. Finally, we showed that isoflavonoids were also exuded in a cluster-root stage-dependent manner, as has been observed for organic acids. However, while the peak of organic acid exudation occurs at the mature stage of cluster roots, we observed that the isoflavonoid peak of exudation took place in the preceding stages, in the juvenile and immature cluster roots. This led us to speculate that these potential antimicrobial compounds may inhibit the soil microflora, and that the burst of phenolics occurring shortly before the exudation of citrate may help to reduce citrate breakdown by rhizosphere bacteria and fungi, a possibility that requires consideration in future studies.

Acknowledgements

We would like to thank Jana Smutny for her excellent technical assistance. We are very grateful to Professor Hans Lambers for his help in improving the English. This work was supported by the Swiss National Foundation within the National Center of Competence in Research 'Plant Survival' and by the Körber Stiftung (Germany).

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Supplementary Material

The following material is available for this article online:

Fig. S1 Electrospray ionization (ESI) mass spectra for O-Glc-Mal-genistein: (a) positive ionization mode [M+H] ion at mass to charge ratio (m/z) 519; (b) negative ionization mode Y_0^- fragment ion at m/z 269 after cleavage of glycosidic bonds. Glc, glucose; Mal, malonyl.

Fig. S2 Nomenclature and diagnostic fragmentation of O-Mal-Glc-genistein. Glc, glucose; Mal, malonyl.

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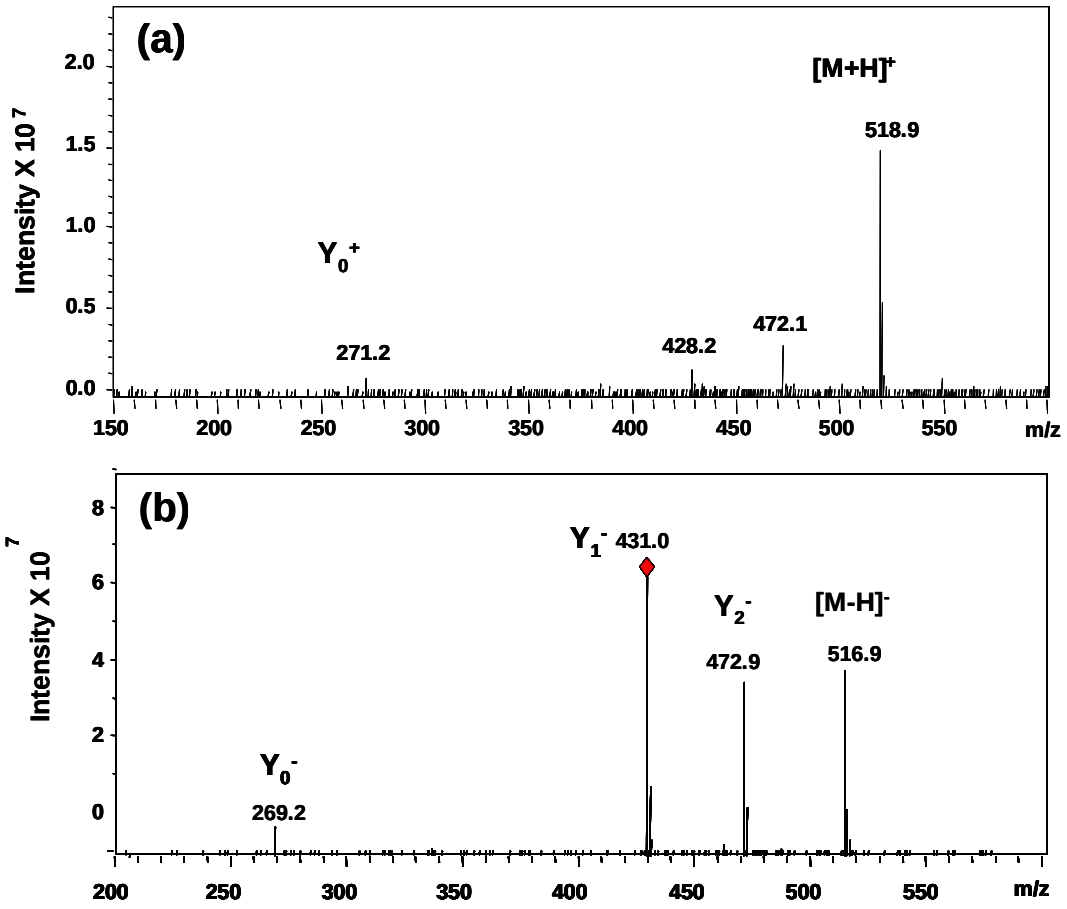
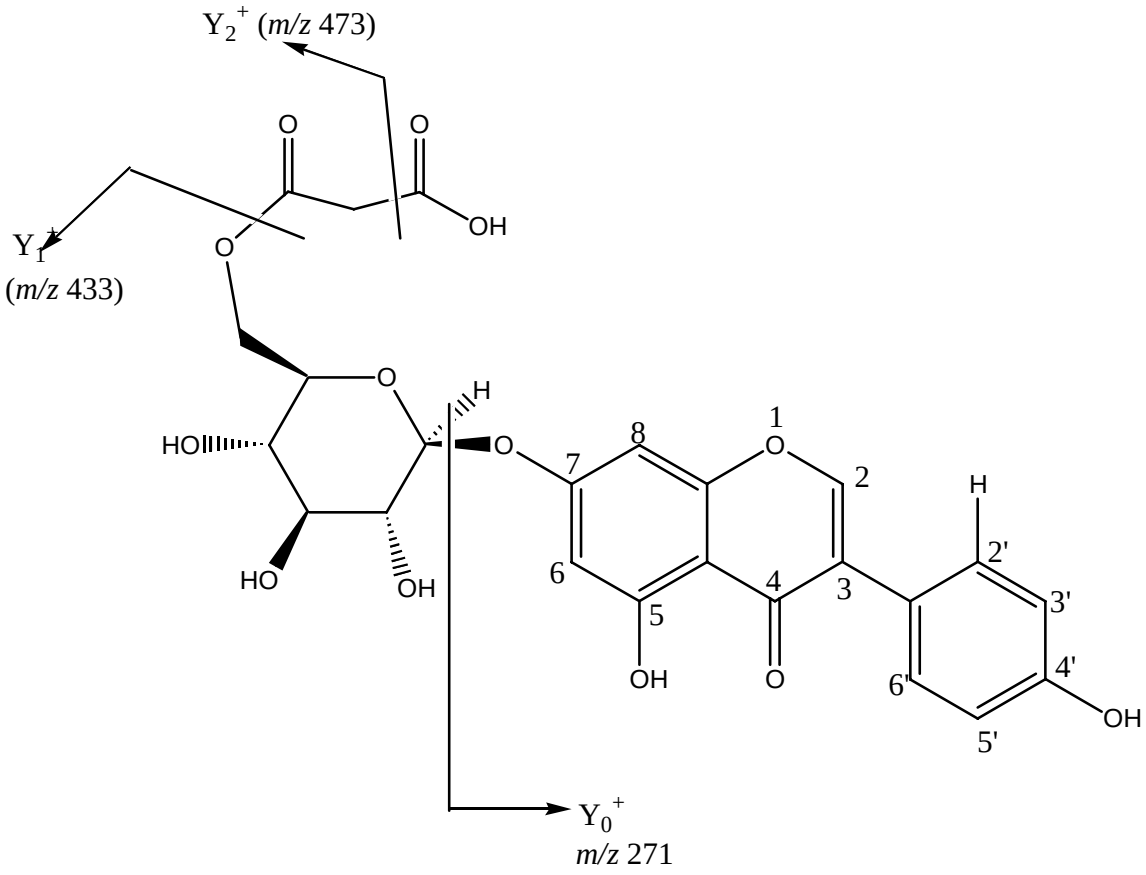
NPH1776: Figure S1



NPH1776: Figure S2



3.2 White lupin has a complex strategy to limit microbial degradation of secreted citrate required for phosphate acquisition

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Reprinted from: Plant, Cell and Environment (2006) **29**: 919-927

We investigated potential protection mechanisms of white lupin against microbial degradation of the phosphate-chelating agents, citrate and malate. We could show that white lupin has developed a complex strategy to protect secreted organic acids which include: i) a decreased abundance of total bacteria around mature cluster roots, due to the strong rhizosphere acidification; ii) an increased activity of glucanase and chitinase at the stage preceding the secretion of citrate; iii) and increased isoflavonoid secretion inducing fungal sporulation in the juvenile and immature stage. Overall, our results indicated that white lupin cluster roots are highly specialized structures that have successfully evolved across evolution, in harmony with the sourronding environment. White lupin only inhibits the microbial rhizosphere populations when it is needed and not in a lasting manner, which would represent a waste of energy and the risk of inhibiting potentially beneficial rhizosphere microorganisms.

Plant, Cell and Environment (2006) **29**, 919–927 doi: 10.1111/j.1365-3040.2005.01473.x

White lupin has developed a complex strategy to limit microbial degradation of secreted citrate required for phosphate acquisition

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ABSTRACT

White lupins (*Lupinus albus* L.) respond to phosphate deficiency by producing special root structures called cluster roots. These cluster roots secrete large amounts of carboxylates into the rhizosphere, mostly citrate and malate, which act as phosphate solubilizers and enable the plant to grow in soils with sparingly available phosphate. The success and efficiency of such a P-acquisition strategy strongly depends on the persistence and stability of the carboxylates in the soil, a parameter that is influenced to a large extent by biodegradation through rhizosphere bacteria and fungi. In this study, we show that white lupin roots use several mechanisms to reduce microbial growth. The abundance of bacteria associated with cluster roots was decreased at the mature state of the cluster roots, where a burst of organic acid excretion and a drastic pH decrease is observed. Excretion of phenolic compounds, mainly isoflavonoids, induced fungal sporulation, indicating that vegetative growth, and thus potential citrate consumption, is reduced. In addition, the activity of two antifungal cell wall-degrading enzymes, chitinase and glucanase, were highest at the stage preceding the citrate excretion. Therefore, our results suggest that white lupin has developed a complex strategy to reduce microbial degradation of the phosphate-solubilizing agents.

Key-words: *Lupinus albus*; chitinase; glucanase; isoflavonoids; phenolics; protection.

INTRODUCTION

In nature, plants often grow in soils where nutrient availability is low. As an adaptation to nutrient deficiency, they have developed several mechanisms. In the case of phosphate deficiency, plants may establish mycorrhizal associations, express high-affinity phosphate transporters or secrete large amounts of carboxylates such as citrate or malate. Secretion of carboxylates in response to phosphate deficiency has been reported for many plant species (Imas *et al*. 1997; Zhang, Ma & Cao 1997; Kirk, Santos & Santos 1999). In addition to this mechanism, some plant species, for example, white lupin and most members of the *Proteaceae* family, form special root structures called cluster or proteoid roots (Purnell 1960; Dinkelaker, Hengeler & Marschner 1995; Neumann & Martinoia 2002; Lamont 2003; Shane & Lambers 2005). These cluster roots strongly acidify the surrounding rhizosphere and secrete large amounts of organic acids, mainly citrate, during a short time span (3–5 d). This strategy enables the plant to efficiently extract Pi from a restricted volume of the soil. Protons solubilize P in calcareous soils. However, white lupin and also most *Proteaceae* naturally grow on acid soils. Dicarboxylates and tricarboxylates act as exchanger anions for phosphate, liberating it from Fe–P, Al–P or Ca–P complexes. The secretion of protons (which probably are exported to avoid cytosolic acidification and to maintain charge balance) and organic acids allows plants that form cluster roots to survive in soils with sparingly available phosphate. However, the efficiency of carboxylate secretion in phosphate acquisition depends to a large extent on the stability and persistence of these compounds in the soil, which is mainly influenced by the biodegradation activity of soil microorganisms. Bacteria and fungi readily take up and metabolize organic acids (Jones, Prabowo & Kochian 1996; Strom *et al*. 2001; van Hees *et al*. 2005). Thus, for plants, an efficient strategy in phosphate acquisition would require an abundant excretion of carboxylates to compensate for the loss by microbial degradation and/or a strategy to limit microbial degradation of organic acids. The latter would imply that plants implement mechanisms reducing growth and viability of micro-organisms in the vicinity of cluster roots. Among the numerous studies devoted to cluster roots and their efficient phosphate-acquisition mechanisms, no report up to now has addressed the question of whether or not plants

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also developed strategies to protect their secreted phosphate-chelating agents from microbial degradation.

We chose to investigate this question by using white lupin as a model plant. This leguminous annual crop has frequently been used to study processes linked to cluster root function (Gardner, Parbery & Barber 1982, 1983; Johnson, Allan & Vance 1994; Neumann *et al*. 1999; Neumann *et al*. 2000; Shane *et al*. 2003; Veneklaas *et al*. 2003; Zhang, Ryan & Tyerman 2004). In white lupin, the formation of the bottlebrush-like cluster roots follows a well-defined developmental pattern (Watt & Evans 1999; Massonneau *et al*. 2001). Young, growing cluster roots release mainly malate, and only small amounts of citrate, while immature cluster roots secrete similar amounts of both. In contrast, mature cluster roots secrete far greater amounts of carboxylates, mainly citrate, and strongly acidify the rhizosphere. Solubilization, soil extraction and uptake of phosphate into the plant occur mainly at this mature stage of cluster root development. Hence, mechanisms developed for the protection of secreted organic acids against microbial degradation are expected mainly at this root stage, or shortly before.

The aim of this study was to investigate if white lupin has strategies to protect secreted organic acids from microbial degradation. We show: (1) a decreased total bacteria abundance around mature cluster roots; (2) an increased isoflavonoid excretion, inducing fungal sporulation, in the juvenile and immature stage; and (3) an increased glucanase and chitinase activity at the stage preceding the excretion of citrate. Our results suggest that white lupin exhibits a complex strategy to protect secreted organic acids against microbial degradation.

MATERIALS AND METHODS

Plant material and growth conditions

White lupin plants (*Lupinus albus* L. cv. Amiga, Südwestdeutsche Saatzucht, Rastatt, Germany) were grown either in microcosms (microbiological analyses) or under hydroponic conditions (phenolics, enzymatic analyses). The microcosms were used in three replicates consisting of one plant planted in one microcosm. Plants were grown in inoculated sand as described previously (Weisskopf *et al*. 2005), except that the inoculation solution was prepared from a lupin field soil collected in Monte Gargano, Foggia, Italy. After 6 weeks of growth, the plants were harvested, and the different root stages were identified with a pH indicator – agar gel method (Weisskopf *et al*. 2005). Juvenile, mature and senescent stages were separated (in soil-grown plants, the distinction between immature and mature cluster roots is not possible). Within a plant (replicate), cluster roots belonging to the same stage were pooled. Roots coming from the different root stages were then washed in a sodium phosphate buffer (SPB) 0.1 M pH 7 , and ground with mortar and pestle. One part of the ground roots was used for isolation and low-pH-tolerance assay of bacterial strains, and the other was prepared for the microscopic count analysis. For phenolics and enzymatic analyses, growth in hydroponic cultures without phosphate supply was carried out as described by Massonneau *et al*. (2001). Three replicates were used, with one replicate consisting of five boxes containing 12 plants each. The different cluster root growth stages were identified by immersing the root system into a pH indicator solution according to Neumann *et al*. (1999). This technique allows the separation of immature and mature roots. Therefore, we were able to separately collect juvenile, immature, mature and senescent cluster roots.

Adsorption and degradation of carboxylates in the soil

Mixtures of organic acids in a concentration range detected in the rhizosphere of cluster roots (malate 7.5 mM; citrate 2.1 mM) and water controls were incubated at 20% soil moisture level with the test soils (250 mg per sample, taken from planted culture vessels to introduce an active soil microflora) using incubation times of 3 and 24 h under axenic and non-axenic conditions. Axenic conditions were obtained by chloroform fumigation of the soil samples with a 24 h pre-incubation period under a chloroform atmosphere. After 3 and 24 h, water-soluble organic acids were re-extracted with 450 μL H_2O. The soil was removed by centrifugation and organic acids in the supernatant were determined by high-performance liquid chromatography (HPLC) (Neumann *et al*. 1999). Recovery of organic acids was calculated in comparison with the water controls. Soil characteristics: Arenosol, West Africa, pH ($CaCl_2$) 4.6; P (CAL) 3 mg kg^{-1}. The influence of the buffer pH on organic acid recovery was analysed using radio-labelled citric acid. Citric acid [30 nCi (^{14}C)], Moravek Biochemicals, Brea, CA, USA, specific activity of 108 mCi $mmol^{-1}$, citrate corresponding to a final concentration of 1 mM] was incubated with 2 g of soil with a moisture of 20% for 1 h. A washing step (incubating the soil with 20 mM phosphate buffer, pH 7 for 20 min with gentle shaking) was carried out twice to eliminate the non-adsorbed citric acid. Extractions were performed with phosphate buffers at pH 7 or 3.2, as well as with 5 mM NaCl and 5 mM HCl. After 30 min of incubation, aliquots of the samples were collected and quantified by scintillation counting. The released citrate was expressed as a percentage of bound citrate. The experiments were repeated thrice, providing similar results.

Isolation of strains and growth in low- pH media

The ground roots from the three different stages of cluster roots were 10-fold serially diluted, and spread on Angle medium (Angle, McGrath & Chaney 1991) for plate counts and isolation of strains. Approximately 30 strains isolated from each cluster root stage were randomly selected and tested for their ability to grow in low-pH media. We prepared LB medium (10 g L^{-1} peptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl) and modified the pH to obtain five different pH values, from 7 to 3. A specific buffer was used for each pH value. The experiment used 0.1 M NaOH (A), 0.1 M $C_8H_5KO_4$ (B), 0.1 M HCl (C) and water (D) for the buffers

in the following proportions (A:B:C:D): 45:50:0:5 for pH 6, 22.6:50:27.4:0 for pH 5, 0:50:0:50 for pH 4 and 0:50:21.6:28.4. Ten millilitres of the corresponding buffer was added to 250 mL of medium prior to autoclaving. The low-pH assay was performed in liquid cultures, using microplates (NUNC, Rochester, NY, USA). Results (growth or no growth) were recorded after 7 d of incubation at room temperature.

Total cell counts after 4′-6-diamidino-2-phenylindole (DAPI) staining

After washing off the rhizosphere soil by gently shaking in SPB, the roots from the three different stages of cluster roots were ground. One millilitre of the ground roots was added to 9 mL of 50% ethanol (v/v) for fixation. The samples were incubated for 4 min in an ultrasonic bath for sample homogenization, final separation of roots from remaining soil and sand particles, and partial dissociation of bacteria from roots. Several incubation times were tested, and the highest recovery of bacteria was obtained after 4 min of sonication. Samples were then centrifuged for 2 min at 500 *g* to allow sedimentation of remaining soil and sand particles. After diluting 1:1 with 50%, ethanol (v/v), 1 mL of the supernatant was filtered on polycarbonate filters (13 mm diameter, 0.2 μm pore size) placed on nitrocellulose filter supports (13 mm diameter, 0.2 μm pore size). The samples were then stained with 70 μL DAPI (5 μg mL^{-1} solution, Sigma, St. Louis, MO, USA) for 5 min in the dark. Excess DAPI was removed, and the filters were observed with a Leica Dialux microscope (Leica Microsystems Ltd, Milton Keynes, UK) at a 1000 × magnification. Cell numbers were determined by counting 15 fields per filter with a grid ocular. The average value of the 15 fields was used for the calculation of the total cell counts g^{-1} root fresh weight (FW).

Extraction of phenolic compounds and HPLC analysis

Excised root parts were washed in distilled water to eliminate the compounds liberated from cut cells, and subsequently incubated in 4 mL of water for 1 h at room temperature with gentle shaking to allow the collection of root exudates. We have previously shown that there is no significant difference in the quality or quantity of exudates released into distilled water compared to those released into 0.5 mM $CaSO_4$, which is often used for membrane stabilization (Neumann *et al*. 1999). We used this hydroponic system to allow the separation and comparison of well-defined cluster root stages, but we were aware that the secreted compounds recovered during this experimental procedure might be different from those obtained in the field conditions, where soil micro-organisms likely influence the root secretions. In order to verify that the pattern of isoflavonoids collected was not affected by microorganisms present in the soil, we analysed the isoflavonoids secreted from lupin seedlings grown in the presence of different bacteria and fungi. The amounts of specific isoflavonoids released changed, but not the composition (data not shown). The root exudates were collected and frozen at –80 °C. The remaining roots were then incubated in 4 mL of 80% methanol (v/v) for 1 h at room temperature with gentle shaking to recover the internal cell contents. Internal isoflavanoid extracts were filtered at 0.45 μm (Schleicher & Schuell, D–37586 Dassel, Germany) and resuspended in the first HPLC solvent according to the root FW (1.5 μL mg^{-1} root FW). The frozen exudates were freeze-dried and subsequently extracted with 2.5 mL of 80% methanol (v/v) in sequential steps (1 mL and subsequently thrice 0.5 mL). Each step was followed by vigorous shaking and filtration at 0.45 μm. After solvent evaporation, exudate extracts were resuspended in the first HPLC solvent in proportion to the root FW (0.75 μL mg^{-1} root FW). Of the samples, 50 μL (internal contents and exudates) were then injected into a reversed phase C 18 column (Nucleosil 250 × 4.6 mm, 7.0 μm) for analysis. To separate the isoflavonoids, we used two solvents consisting of water, acetonitrile and acetic acid in the following proportions: 93:5:2 (A) and 23:75:2 (B). Solvent gradient started with 10% solvent B and reached 100% B in 25 min, with a flow rate of 0.4 mL min^{-1}. The absorbance was monitored at 263 nm. All analyses were performed with three to four replicates, with each replicate representing the harvest of five boxes containing 12 plants each.

Flavonoid staining with diphenyl-boric acid dimethyl amino-esther (DPBA)

The entire root system of white lupin plants grown in hydroponic culture under P-deficient conditions was placed between two agar gel layers containing 5‰ of DPBA (Sigma). After 20 min of incubation in the dark, the stained root parts were investigated under UV light (360 nm) exposure. The same staining procedure was applied to single roots.

Effects of isoflavonoids on the sporulation of *Fusarium oxysporum*

Phenolic compounds [100 μg in ethanol in 20 μL 50% ethanol (v/v)] were separated on thin-layer chromatography (TLC) plates (Silica gel 60 F254, Merck, Darmstadt, Germany) in a dichloromethane/methanol mixture (95:5) and one-half of the plate was used for isoflavonoids staining with 1% Gibbs reagent in ethanol. The solvent [50% ethanol (v/v)] was used as a control. A spore suspension of the lupin pathogen *F. oxysporum* was spread on the other half of the plate. The spore suspension was prepared by mixing 1 mL of sterile water containing spores collected on a Petri dish and 10 mL of melted potato–dextrose–agar (PDA) 10-fold diluted and containing half the agar concentration to allow a thin and easy spreading on the plate.

Enzymatic assays of chitinase and glucanase

For the analysis of enzymatic activities, plants were grown in hydroponic culture under P-deficient conditions. Four to seven replicates were used, consisting of one box containing 12 plants. After the separation of the different cluster root stages, excised root samples were incubated for 30 min at room temperature in 5 mM $CaCl_2$ (pH 6.4) with gentle shaking. The extracellular fraction was collected, 1 mg mL^{-1} bovine serum albumin (BSA) was added and samples were frozen at –80 °C prior to lyophilization. The lyophilized samples were resuspended in a 0.1 M sodium acetate buffer pH 5.

Chitinase activity

Chitinase activity was determined with dye-labelled 6-0-carboxymethyl-chitin (chitin azure, Sigma) according to Wirth & Wolf (1990) after optimization of incubation time and enzymatic extract concentration. Each reaction mixture contained 0.2 mg chitin azure, 500 μL enzymatic extract and 200 μL 0.1 M sodium acetate buffer pH 5. For each sample, a blank sample containing no chitin azure was also prepared to take into account the colour differences of the different root stages. After 1 h of incubation at 30 °C with gentle shaking, 200 μL 0.1 M HCl was added to stop the reaction, and the samples were centrifuged for 5 min at 1800 *g*. The absorbance was read at 560 nm. For each sample, the absorbance value of the negative control (lacking chitin azure) was deducted from the final absorbance. Chitinase activity was expressed as the absorbance at 560 nm root FW^{-1} h^{-1}.

Glucanase activity

Glucanase activity was determined by measuring the release of glucose from laminarin. Each reaction mix contained 100 μL of laminarin solution (18 mg mL^{-1} in sodium acetate buffer pH 5 0.1 M) and 200 μL enzymatic extract. For each sample, a blank sample without laminarin was used to account for the colour changes between different root ages. After 1 h of incubation at 30 °C with gentle shaking, 150 μL was collected, and released glucose was determined using the Boehringer Mannheim D-Glucose determination kit (Boehringer Mannheim, Germany). Glucanase activity was expressed as mg glucose g^{-1} root FW h^{-1}.

Statistical analyses

Microscopic counts were compared statistically using Student's *t*-test ($P < 0.01$). The results of bacterial growth in low-pH media were validated using a Chi-square test (χ^2-test) ($P < 0.05$). For phenolic contents and enzyme activities, Student's *t*-test was used to validate differences ($P < 0.05$). These analyses were performed using S-Plus 6 Statistical Software (Insightful Corporation, Seattle, WA, USA).

RESULTS

Carboxylates are adsorbed to the soil and degraded by micro-organisms

In order to test whether carboxylates are rapidly degraded by soil micro-organisms, we added malate and citrate to an acidic soil in concentrations reported for the rhizosphere soil of cluster roots. As shown in Table 1, most of the citrate and malate were adsorbed to the soil matrix. Adsorption was differentially expressed for malate and citrate, and accounted for 55–70% of the recovery of carboxylates. After 3 h, the same recovery was obtained under axenic and non-axenic conditions, indicating that at this time scale, microbial degradation was negligible. Moreover, carboxylate concentrations in the soil solution rapidly reached equilibrium concentrations, because recovery after 3 and 24 h axenic incubation revealed similar values. This is in line with observations of Jones *et al*. (2003).

The impact of biodegradation on carboxylate recovery increased with incubation times longer than 3 h, leading to complete degradation of the extractable organic acids within 24 h, even at the high concentration levels accumulating in the rhizosphere of cluster roots. Because it must be assumed that an equilibrium between bound and free carboxylates is established, microbial degradation will result in partial release of bound carboxylates, and consequently, biodegradation is probably underestimated. Because white lupin acidifies the rhizosphere, we tested if the use of an acidic buffer (pH 3.2) would release more organic acids. This was indeed the case (about 6% at pH 7 and 12% at pH 3.2), increasing the organic acid fraction

Table 1. Recovery of water-extractable citrate and malate after incubation in axenic versus non-axenic conditions

	Recovered by water extraction (%)			
Carboxylates	3 h axenic	3 h non-axenic	24 h axenic	24 h non-axenic
Malate (7.5 mM)	46.7 ± 4.0	44.5 ± 4.2	34.8 ± 1.7	0
Citrate (2.1 mM)	32.5 ± 3.5	29.5 ± 1.9	12.8 ± 11.1	0

Recovery of water-extractable organic acids applied in concentrations reported for the rhizosphere soil solution of cluster roots (Neumann & Römheld 2000) at a soil moisture level of 20% and different incubation times under axenic (chloroform atmosphere) and non-axenic conditions. Means and SD of three independent replicates.

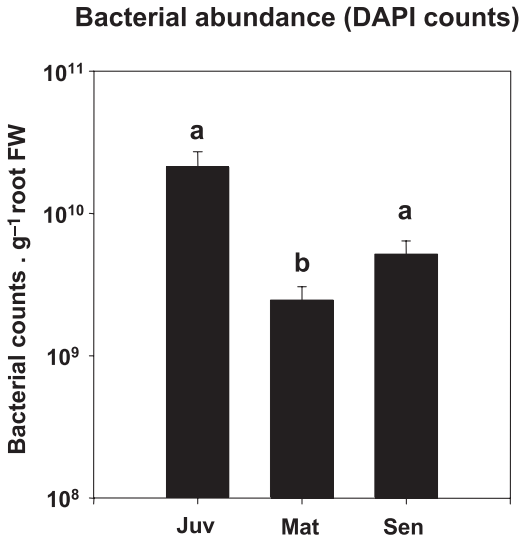

Figure 1. Numbers of bacteria g^{-1} fresh weight (FW) as a function of cluster -root stage. Bacterial abundance was determined by microscopic counts after DAPI staining.
Juv, juvenile; Mat, mature; Sen, senescent. Values with different letters (a, b) are significantly different (Student's *t*-test, $P < 0.01$, $n = 3$).

available for microbial degradation. These findings emphasize the importance of mechanisms that counteract the biodegradation of carboxylates for an efficient P-mobilization, which usually requires mM concentrations of carboxylates in the extraction solution (Neumann & Römheld 2000).

Acidification at the mature cluster root stage decreases bacterial abundance

In a recent paper (Weisskopf *et al*. 2005), we showed that the number of bacteria that could be cultivated was significantly reduced at the mature stage of cluster roots. In order to verify if this reduced abundance deduced from plate counts was not a result of a decrease in the capacity of the different bacteria to be cultivated, we performed microscopic counts after DAPI staining of bacteria collected at different root stages. In agreement with what was observed for bacteria that could be cultivated, total bacterial abundance decreased significantly at the mature stage of cluster roots, which corresponds with the stage of fastest citrate excretion (Fig. 1).

A possible explanation for this decrease could be the low pH (4 or below) associated with the mature stage of cluster roots, because of a concomitant release of protons and citrate excretion. To test this hypothesis, we assessed the ability of isolated strains to grow at pH conditions ranging from pH 3 to 7. The ability of about 100 strains isolated from three stages of white lupin cluster roots to grow on an acidic medium (pH 4) is shown in Fig. 2. The proportion of strains able to grow at pH 4 was significantly higher for mature stage cluster roots than for the juvenile stage ones, suggesting that because of the transient acidification of the rhizosphere, a selection of acid-tolerant populations had taken place at the mature stage of cluster roots, whereas the more sensitive populations had been inhibited. At pH 3, no bacterial growth was observed, and no significant differences among the different stages were observed at the other pH values (data not shown).

Prior to citrate excretion, isoflavonoids are secreted in large amounts into the rhizosphere

Because fungi are more tolerant to acidic pH compared to bacteria, we investigated other possible defence mechanisms involved in fungal growth inhibition. It has been reported previously that cluster roots secrete phenolics (Neumann *et al*. 1999), which might act as antifungal compounds. The white lupin cluster roots secreted large amounts of phenolic compounds, and most of these phenolics were isoflavonoids. The internal concentration of isoflavonoids significantly increased from the juvenile to the immature stage (Fig. 3a). The excretion pattern followed the same trend with the largest amounts secreted at the juvenile and immature stages, the root stages preceding the citrate burst (Fig. 3b). Genistein was the isoflavonoid secreted in largest amounts (up to 0.8 mg g^{-1} root FW h^{-1} from immature cluster roots, data not shown), and most other compounds were conjugates of genistein (Weisskopf *et al*., unpublished results).

This pattern of isoflavonoids in the different stages of white lupin cluster roots was confirmed by an *in situ* staining of the entire root system with DPBA, a flavonoid-specific dye (Fig. 3c). Young and immature cluster roots

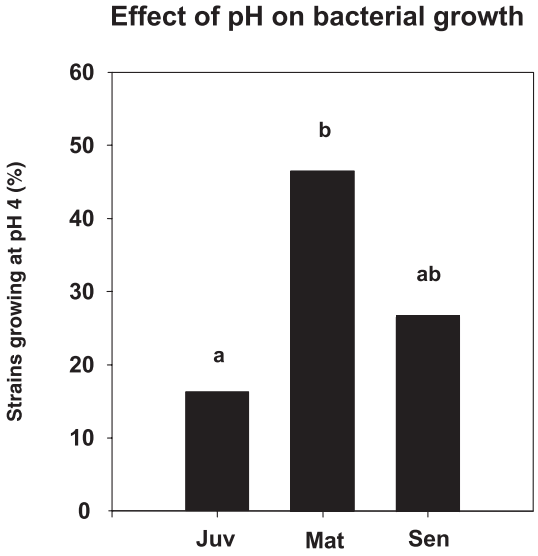

Figure 2. Frequencies (in percentage of total isolated strains) of strains growing in a low-pH (4) medium. About 100 strains were randomly picked, at least 30 per root stage.
Values with different letter (a, b) are significantly different [(Chi-square test (χ^2-test), $P < 0.05$, $n \geq 30$]).
Juv, juvenile; Mat, mature; Sen, senescent.

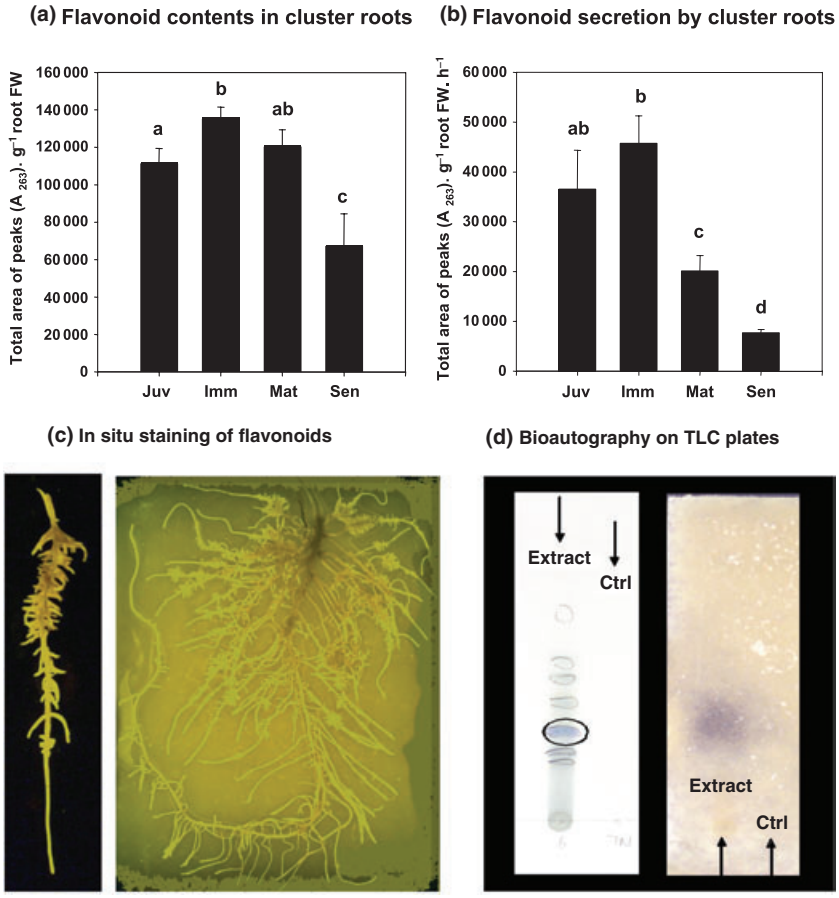

Figure 3. Flavonoids in white lupin cluster roots. Flavonoids produced in (a) and secreted from (b) different stages of cluster roots. Juv, juvenile; imm, immature; mat, mature; sen, senescent. Total amounts were calculated as the total area of all peaks measured by UV detection (263 nm) after high-performance liquid chromatography (HPLC) analysis. Values are means of three replicates, one replicate consisting of five boxes containing 12 plants each. Different letters (a, b, c, d) represent significantly different values (Student's *t*-test, $P < 0.05$, $n = 3$). (c) *In situ* staining of flavonoids with DPBA (diphenyl-boric acid dimethyl amino-esther (DPBA)). White lupin roots (single cluster roots: left picture; whole root system: right picture) were placed between two agar gel layers containing 5‰ DPBA. The brightness reflects the amounts of flavonoids present in the root tissues, and secreted at the surface. After 20 min of incubation, the roots were exposed to UV light (360 nm). (d) Effects of flavonoids secreted by white lupin cluster roots on the sporulation of *Fusarium oxysporum*. Left panel: 50 μg of phenolic extracts were separated on a thin-layer chromatography (TLC) plate, and stained with the flavonoid-specific Gibbs reagent. One compound (blue) was highly absorbing UV light (circle). Right panel: Inoculation of the TLC plate with a spore suspension of *F. oxysporum* showed an enhanced sporulation at the same location as the highly absorbing compound, identified as malonyl genistein. A 50 μg part of phenolic extracts was used for this assay. Ctrl: negative control (only solvent). Picture was taken 5 d after inoculation. See Material and Methods section for details.

exhibited a brighter fluorescence than mature or senescent cluster roots. This was also true for non-cluster roots, where mostly the apex and growing parts were stained. Because isoflavonoids are well-known antifungal compounds, one could imagine that this observed 'phenolic burst' prior to citrate exudation would exhibit an inhibitory action on fungal growth, and hence also contribute to the protection of citrate from microbial degradation. To test this hypothesis, we performed *in vitro* assays with the bulk of secreted phenolics. We tested about 20 morphotypes of fungi isolated from white lupin rhizosphere, as well as some collection strains, which represented potential pathogens of white lupin. Among the tested fungi, some showed an increased sporulation when exposed to the phenolics secreted by white lupin. Because active growth and nutrition occur in fungi only during the vegetative stage (mycelium) of the life cycle, we can assume that at the sporulated stage, fungi will not consume any citrate. One collection strain belonging to the species *F. oxysporum* showed the strongest reaction, and was chosen as a biotest strain for further investigations. In a bioassay based on TLC plate bioautography (Fig. 3d), we observed that stimulation of sporulation occurred approximately at the height (Rf) as the isoflavonoid with the highest UV absorption, identified as malonyl genistein. Unfortunately, we were not able to observe this stimulation of the sporulation when fractions were fur-

ther purified, suggesting that more than one compound migrating at a similar Rf value as malonyl genistein were involved in this phenomenon, and that they might act in a synergistic way. No inhibitory effect of these isoflavonoids on bacterial growth was observed.

Chitinase and glucanase show higher activities in the stage immediately preceding high citrate excretion

A widespread defence mechanism of plants against a broad spectrum of fungi is the secretion of cell wall-degrading enzymes into the rhizosphere. We have shown in a previous study that with a complementary DNA amplified fragment length polymorphism (cDNA–AFLP) assay, several genes involved in carbohydrate metabolism were differentially expressed during cluster root formation under P-deficient conditions (Massonneau *et al*. 2001). Later analysis of the cDNA–AFLP data showed that among the genes differentially expressed, there were two antifungal enzymes, a glucanase and a chitinase (not shown), as potential candidates to be highly induced during cluster root formation. The sequences of these genes were clearly plant-derived, and did not correspond to microbial chitinases. To verify that the higher level of gene induction corresponded to an enhanced activity, we measured extracellular glucanase and chitinase activities at the different cluster root stages. Chitinase and glucanase activities were both significantly ($P < 0.05$) higher in the immature stage of cluster roots (Fig. 4) compared to all other stages.

The cultivation technique used keeps microbial growth very low. This is reflected by the fact that secreted malate and citrate does not significantly decrease when keeping the samples for 60 min at room temperature, excluding the fact that microbial chitinases influenced the measured enzymatic activities. Furthermore, a semiquantitative reverse transcriptase-PCR for glucanase transcripts revealed a similar pattern as the enzymatic activities (not shown). These results show that the release of chitinase and glucanase also followed a developmental pattern similar to organic acid and isoflavonoid release.

DISCUSSION

In natural environments, phosphate is often a factor that limits plant growth. To deal with this situation, most plants form mycorrhizal associations, which enable them to survive in soils with sparingly available phosphate. An alternative, very efficient strategy to mobilize phosphate from soils and is used mainly by non-mycorrhized plants, is the formation of proteoid roots. In contrast to the phosphate-acquisition strategy of mycorrhizae, cluster root-bearing plants secrete large amounts of carboxylates in a restricted volume of soil at a short time span. The carboxylates serve as exchange anions to solubilize sparingly available phosphate in proximity of the rootlets. The problem of this strategy could be that micro-organisms might efficiently take up and metabolize carboxylates. There was no significant difference between juvenile, mature and senescent cluster root stages when comparing the percentage of isolated strains that are able to use citrate as a carbon source, but overall, these percentages were very high (85% on average, data not shown). For plants forming cluster roots (secreting organic acids), it is therefore crucial to limit the breakdown of organic acids by microorganisms. Several mechanisms can lead to an inhibition of microbial growth. However, different strategies have to be used for bacteria and fungi. Bacteria, in general, are more sensitive to acidic environments than fungi, and a transient decrease of the rhizosphere pH should limit their activity. This transient pH decrease is precisely what happens at the mature stage of

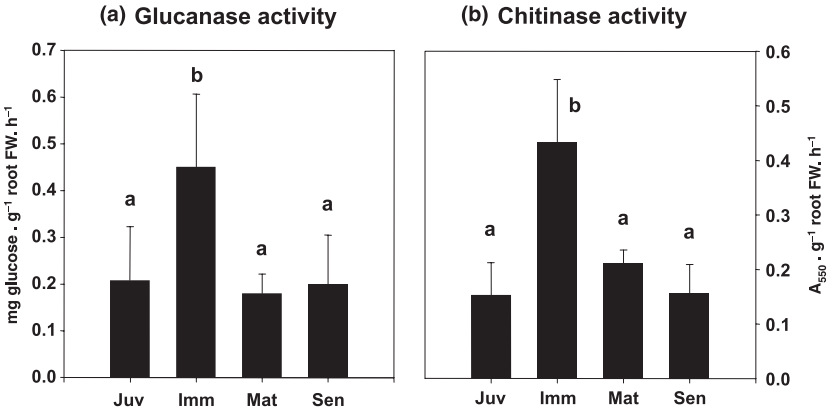

Figure 4. Activities of cell -wall- degrading enzymes at the different cluster -root stages. Juv, juvenile; imm, immature; mat, mature; sen, senescent. (a) Glucanase activity was measured spectrophotometrically by determining the amount of glucose liberated from laminarin during a one-hour incubation period. (b) Chitinase activity was measured spectrophotometrically using the dye-labelled chitin -azure for a one-hour incubation period.
Values are averages of four to seven replicates.
Values with different letters (a, b, c, d) are significantly different (Student's *t*-test, $P < 0.05$, $n = 4–7$).

cluster roots, which can acidify the rhizosphere to a pH of 4 and below. We showed that the bacterial population levels transiently decreased at the mature stage of cluster roots. Furthermore, the mature stage of cluster roots harbored a significantly higher proportion of strains able to grow in acidic conditions (pH 4) than the other cluster root stages. This indicates that the strong acidification occurring at the mature stage of cluster roots could be responsible for the decrease in bacterial abundance associated with this stage, and that only acid-tolerant populations are able to grow in the vicinity of mature cluster roots, whereas the more sensitive populations are transiently inhibited. Thus, the proton extrusion occurring concomitantly with the excretion of organic anions may have several roles: (1) participating in the maintenance of a charge balance through compensation of the secreted negatively charged citrate (Neumann *et al*. 1999; Zhu *et al*. 2005); (2) solubilizing P in calcareous soils; (3) providing an optimal pH for the secreted hydrolases, such as chitinase, glucanase or acid phosphatase, and as suggested by this study; (4) transiently decreasing the bacterial density in the rhizosphere and reducing their degradation of the phosphate-chelating agents.

Heterotrophic soil fungi also can use organic acids as carbon source, and could thus also contribute to a decrease in the P-acquisition efficiency of white lupin cluster roots. Because fungi usually are better able to cope with acidic conditions, other mechanisms than rhizosphere acidification are required to inhibit fungal growth. Some phenolics, like flavonoids or isoflavonoids, display antifungal activities (Tahara *et al*. 1994; Dakora & Phillips 1996; Weidenborner & Jha 1997). White lupin produces a large amount of different isoflavonoids (Stobiecki *et al*. 1999; Katagiri, Ibrahim & Tahara 2000) and some of them act as antifungal compounds (Wojtaszek & Stobiecki 1997; Bednarek *et al*. 2003). We found that isoflavonoids are secreted from cluster roots of white lupin. We also showed that white lupin isoflavonoids induced sporulation in several fungal strains. Sporulation is often a stress response in fungi, and because spores, in contrast to mycelium, do not cause degradation of citrate and malate, this stimulation of sporulation can be viewed as a mechanism to protect the organic acids involved in phosphate acquisition. One could argue that fungi may in turn have a higher nutritional demand for the spore formation, and thus may have consumed a large amount of organic acids in order to sporulate. However, the fact that the isoflavonoid burst occurs prior to the citrate excretion suggests that sporulation takes place before organic acids are secreted and could be consumed. When spores germinate, the newly developed mycelium could then again degrade organic acids. However, it is unlikely that this occurs at the short time span of cluster root development and organic acid secretion (2–4 d). Interestingly, among the fungi tested, *F. oxysporum* was the most susceptible to white lupin isoflavonoids. *Fusarium* species have been previously reported to be inhibited by flavonoids (Silva, Weidenborner & Cavaleiro 1998), and to elicit isoflavonoid accumulation in soybean roots (Lozovaya *et al*. 2004). Furthermore, *Fusarium* species are well-known pathogens of lupins (Bateman 1997; Satyaprasad, Bateman & Ward 2000; Shield *et al*. 2000).

In addition to this increased isoflavonoid excretion, white lupin apparently uses a second, and surely more generally efficient way to inhibit fungal growth: extracellular enzymes like chitinase and glucanase. Both chitinase and glucanase have been shown to play a role in plant defence against pathogenic fungi, in white lupin as well as in other plant species (Vierheilig *et al*. 1994; Burzynski, Pislewska & Wojtaszek 2000; Tonon *et al*. 2002). However, because these enzymes are involved in fungal cell wall degradation, their inhibitory effect will not only be limited specifically to pathogenic fungi, but will also affect other rhizosphere fungi, which may use citrate and malate as carbon sources. In white lupin cluster roots, we showed that both chitinase and glucanase had a higher activity at the immature stage, the stage preceding the rapid citrate excretion.

In conclusion, our results suggest that white lupin exhibits a complex strategy to protect secreted organic anions from microbial degradation: acidification against bacteria and excretion of isoflavonoids as well as cell wall-degrading enzymes against fungi.

ACKNOWLEDGMENTS

We would like to thank Jana Smutny for excellent technical help, as well as Prof. Geneviève Défago and Miroslav Svercel for supplying the fungal collection strains. We are very grateful to Prof. Hans Lambers for critically reading the manuscript and correcting the English. E.M. wishes to dedicate this work to the late Ettorina Pezzoli. This work was supported by the Swiss National Foundation within the National Center of Competence in Research 'Plant Survival' and by the Körber Stiftung (Germany).

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Received 6 September 2005; Accepted 23 September 2005

3.3 P-responsive MATE genes in white lupin roots are possible candidates for isoflavonoid and citrate secretion

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Upublished data

Based on recent evidence from the literature, suggesting that members of the MATE transporters family might be involved in organic acids and/or flavonoids transport, we investigated whether the P-sensitive lupin MATE genes, *LaMATE1* and *LaMATE2*, could be candidates for this transport processes.

To test this hypothesis we characterized the gene expression profiles and found that both genes were upregulated specifically in the mature stage of cluster roots, where solubilization, soil extraction and uptake of phosphate into the plant occur. We localized the closest *Arabidopsis* homolog (AtDXT12) to LaMATE2 at the plasma membrane where also LaMATE1 was shown to be localized. Unfortunately, technical problems encountered during the molecular work and the fact that white lupin was reported until very recently not to be transformable, limited the output of this work, which therefore remains in its infancy. It anyway represents a promising starting point for the characterization of the transport functions of the two lupin MATE transporters.

P-RESPONSIVE MATE GENES IN WHITE LUPIN ROOTS ARE POSSIBLE CANDIDATES FOR ISOFLAVONOID AND CITRATE SECRETION.

Introduction

Plants have developed adaptive mechanisms that aid in the acquisition of phosphorus (P) from soil. Compared to many plant species, white lupin (*Lupinus albus* L.) displays extreme tolerance to P deficiency (Neumann and Martinoia, 2002). Its adaptation to low P is a highly coordinated modification of root development and biochemical adaptations resulting in cluster roots, which are densely clustered secondary roots of determinate growth (Johnson et al., 1996; Massonneau et al., 2001). Cluster roots strongly acidify the surrounding rhizosphere and secrete large amounts of organic acids during a short time span (three to five days). The secretion of protons solubilizes Pi in calcareous soils whilst in acidic soils, di- and tricarboxylates act as anion exchangers for rock phosphate, liberating phosphate from Fe-Al-P complexes (Jones and Darrah, 1994; Ryan et al., 2001b). Formation of cluster roots in white lupin follows a well-defined developmental pattern (Massonneau et al., 2001). Young, growing cluster roots release malate and low amounts of citrate while immature cluster roots excrete comparable amounts of citrate and malate. In contrast, mature cluster roots excrete higher amounts of carboxylates, mainly citrate.

Exudation of organic carboxylates by cluster roots in response to nutrient stresses could result from altered organic anion metabolism, as indicated by enhanced *in vitro* activities of PEP carboxylase and malate dehydrogenase or reduced activity of aconitase (Neumann and Romheld, 1999) and ATP-citrate lyase (Langlade et al., 2002). However, the correlation between the internally produced amounts of citrate and citrate efflux rates from these roots is unclear. For example, mature and senescent cluster roots contain comparable amounts of citrate but only the mature roots actively secrete citrate (Neumann and Romheld, 1999). Therefore, further mechanisms operating independently from citrate and malate synthesis might exist. Recently, an energetically passive citrate efflux through citrate-permeable anion channels has been proposed to be operative in lupin cluster roots (Zhang et al., 2004). Among the currents identified in the plasma membrane of active proteoid roots, IRAC (inwardly rectifying anion channel) was shown to be inwardly rectifying anion channels, supporting anion fluxes and exhibiting

permeabilities which could account for citrate efflux (Zhang et al., 2004). However, the macroscopic IRAC conductance is comparable in protoplasts isolated from phosphate-starved cluster- and non-cluster roots and in protoplasts isolated from phosphate-supplied root apices (Zhang et al., 2004). This is inconsistent with the large, phosphate- and tissue-dependent differences in organic anion exudation. Moreover, the analysis of the correlation between cation and anion exudation in proteoid roots demonstrated that malate release strictly correlates with H^+ extrusion while electroneutrality of citrate efflux may also be provided by K^+ or Na^+ (Zhu et al., 2005), suggesting independent transport systems for malate and citrate, which could involved the activity of plasma membrane transporters, such as the MATE transporters.

Indeed, an abstract submitted by T.P. Durrett, W. Gassmann and E.E. Rogers from the University of Missouri for the 2006 meeting of the American Society of Plant Biologists (http://abstracts.aspb.org/pb2006/public/P11/P11006.html) reports on the potential role of the *Arabidopsis* MATE transporter FRD3 in citrate loading into the xylem. The *frd3* mutant which exhibits constitutive expression of plant strategy I responses to iron deficiency (high proton release into the rhizosphere, Fe(III) chelate reductase activity and Fe(II) transport activities), exhibits reduced xylem exudate levels of iron and citrate. In growth media supplied with citrate, the chlorotic phenotype of *frd3-1* plants is rescued. Preliminary two-electrode voltage clamp data revealed that FRD3 expressed in *Xenopus laevis* oocytes mediates currents when exposed to citrate. These results support the hypothesis that FRD3 effluxes an organic acid, most probably citrate, from the cytosol into the apoplast.

Besides organic acids, cluster roots also secrete phenolics, mainly isoflavonoids (Neumann et al., 2000). We have previously shown that white lupin roots secrete more isoflavonoids – especially more genistein – when the plants grow in the absence of phosphate (Weisskopf et al., 2006a; Weisskopf et al., 2006b). Cluster and non-cluster roots show the same pattern of isoflavonoid secretion, except that higher amounts are secreted from cluster roots than from non cluster roots, suggesting that the higher secretory activity of cluster roots is not restricted to carboxylates only but probably extends to secondary metabolites. Moreover, isoflavonoids like carboxylates are secreted in a cluster root stage-dependent manner. Whilst the peak of organic acid secretion occurs at the mature stage of cluster roots, the isoflavonoid peak of secretion is in the preceeding stages, in the juvenile and immature cluster roots. Functions of root flavonoids are not well understood. However, it is generally accepted that flavonoids

secreted into the rhizosphere are efficient antifungal or antibacterial compounds (Dakora and Phillips, 1996). Furthermore, isoflavonoid secretion by various species of the Leguminosae is considered as a classical phytoalexin response synthesized in response to pathogen attack (Dixon and Paiva, 1995). As a consequence, the higher amount of isoflavonoids secreted during phosphate starvation may represent an evolutionary adaptation which allows the plant to effectively control microbial growth in the rhizosphere. In turn, root-secreted organic acids would not be used as a carbon source for microorganisms and could exclusively act in phosphate acquisition (Weisskopf et al., 2006a).

While the importance of flavonoid excretion from roots is evident, the nature of the transporters is still unclear. Vacuolar transport studies indicate the co-existance of either ABC-type transporters or H^+-antiporters depending on the conjugation pattern (Frangne et al., 2002; Klein et al., 2002; Klein et al., 2000). In contrast, plasma-membrane transport of flavonoids is not investigated at all. The recent identification of the *TRANSPARENT TESTA12* gene in *Arabidopsis* which encodes a MATE transporter that is proposed to be involved in the vacuolar deposition of proanthocyanidin precursors in testa cells (Debeaujon et al., 2001), suggests that this family of transporters could be involved in flavonoid transport. In several cases, MATE proteins have been shown to facilitate the H^+- or Na^+-coupled cellular efflux of small organic molecules (Omote et al., 2006).

Based on this information, we decided to investigate whether lupin *MATE* genes exist that are specifically upregulated in cluster roots under phosphate stress and whether two resulting candidates, the P-sensitive *LaMATE1* and *LaMATE2* genes in white lupin roots could be involved in flavonoid and/or citrate transporter. *LaMATE1* was first identified by a functional genomic approach (Uhde-Stone et al., 2003), and later shown to be specifically induced by nutrient deficiencies, such as phosphorus, nitrogen and manganese; the protein was localized at the plasma membrane of transgenic white lupin proteid roots cells (Uhde-Stone et al., 2005). *LaMATE1* displays homology to FDR3 in *Arabidopsis*, but it did not complement the *frd3* mutant (Uhde-Stone et al., 2005). *LaMATE2* was cloned in our laboratory after having been identified as an abundant gene in a cDNA-AFLP approach. Prelimary data on *MATE* gene expressions and subcellular localization suggest a role for these proteins in the efflux of small organic molecules.

Results

LaMATE2 is the homolog of the putative MATE protein DTX12 from **Arabidopsis thaliana.**

In order to identify stage-specific expression of mRNA, a cDNA-AFLP analysis with juvenile, mature and senescent cluster roots was previously performed in our laboratory (Massonneau et al., 2001). Among the 60 sequences identified, a cDNA clone corresponding to a putative MATE related sequence was isolated. We sequenced and analyzed the full-length cDNA clone. The 2-Kb cDNA contained a 1464 bp open reading frame (ORF) that encodes a deduced protein of 487 AA with a predicted molecular mass of 53.7 kD and a calculated pI of 7.96. Hydropathy analysis predicts that proteins of the MATE family have a common topology consisting of 12 transmembrane (TM) domains (Li et al., 2002). Consistently, LaMATE2 prediction of transmembrane domains by TopPred (Claros and Vonheijne, 1994) indicated that the protein contains 12 putative transmembrane domains (TMDs) connected by hydrophilic loops of various size and that the N and C hydrophilic termini are located in the cytosol (Figure 1A-B).

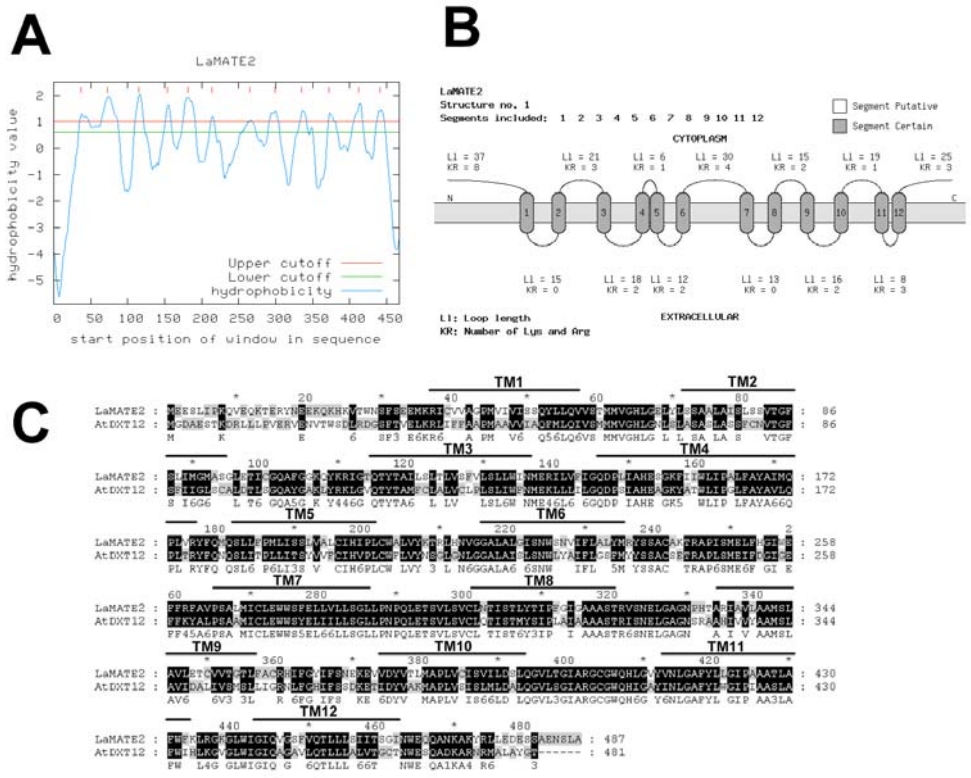

Figure 1. LaMATE2 protein sequence analysis. Hydrophobicity pattern **(A)** and TMs domain **(B)** prediction of the deduced amino acid sequence of LaMATE2. **(C)** Alignment of LaMATE2 and AtDXT12 protein sequences.

This membrane topology is indeed characteristic for many transporter proteins found in both prokaryotes and eukaryotes (Henderson, 1993). Interestingly, LaMATE2 is predicted to localize to the plasma membrane according to TargetP (Emanuelsson et al., 2000). However, these data are only *in silico* predictions based on analyses of protein sequences and must therefore be considered cautiously, especially since no clear targeting signals for membran proteins destined to reach the tonoplast are identified.

A comparison of the predicted LaMATE2 protein with sequences in various databases was performed with Gapped Blastp server (http://www.ncbi.nlm.nih.gov/BLAST/), yielding representatives from all three taxonomic kingdoms. The best similarity was found with the putative MATE protein DTX12 in *Arabidopsis* (At1g15170, Li et al., 2002) sharing 62 % identity and 80 % similarity of the AA sequence (Figure 1C). Like LaMATE2, DTX12 is also predicted to possess 12 transmembrane domains (data not shown).

Nutrient deficiencies induce LaMATE gene expression

LaMATE1 was previously shown to be highly expressed in –P stressed proteoid roots and to a lesser extent in –P normal roots; no expression was detected in +P lupin (Uhde-Stone et al., 2003). Hower, analysis of *LaMATE1* gene expression in different developmental stages of cluster roots has not been reported to date. Here, semi-quantitative RT-PCR analysis starting with RNA extracted from different stages of cluster root development and from P supplied roots was performed for both *LaMATE* genes. *LaMATE1* transcript levels were maximal in mature cluster roots and decreased significantly in the senescent cluster roots. In juvenile cluster roots *LaMATE1* was not detected (Figure 2A). Like *LaMATE1*, *LaMATE2* transcription was induced upon P starvation and mRNA levels were also highest in mature cluster roots. Expression was still high in senescent cluster roots (Figure 2B). The strong induction of both *LaMATE* genes at the mature stage of cluster roots, correlating with maximal secretion of citrate, points to a possible role for MATE transporters in the control of the efflux of small organic molecules, such as citrate.

Expression of *LaMATE1* in lupin is influenced by other nutritional stresses besides –P, such as nitrogen (-N), iron (-Fe), manganese (-Mn) and aluminium (+Al) (Uhde-Stone et al., 2005). To investigate whether *LaMATE2* expression was also induced by other stresses, semi-quantitative RT-PCR was performed using RNA samples of normal and

proteoid roots from white lupin grown in the absence of iron (-Fe). Indeed, *LaMATE2* mRNA is induced in all developmental stages of cluster roots upon iron starvation (Figure 2B). Again, highest mRNA levels were found in the mature cluster roots.

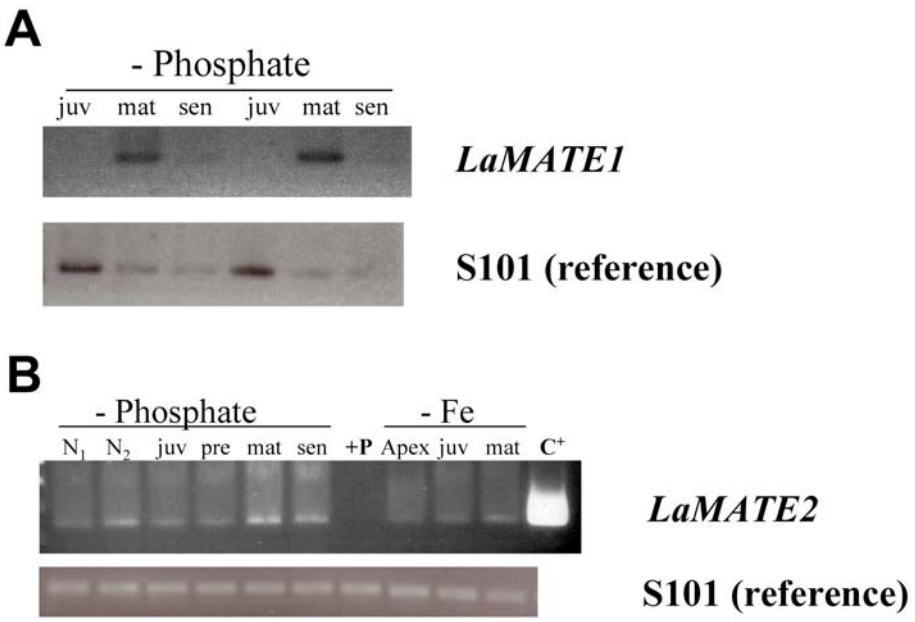

Figure 2. LaMATE1 and LaMATE2 RT-PCR in different white lupin cluster root stages. Root tissue expression pattern are presented for *LaMATE1* **(A)** in - P and for *LaMATE2* **(B)** in ± P and ± Fe conditions. As a control, the expression pattern of the ribosomal protein S101 is also presented. Juv, juvenile; Pre, premature; Mat, mature; Sen, senescent.

AtDTX12 is localized at the plasma membrane

To obtain further insight into the mechanism of MATE protein function we investigated the subcellular localization of LaMATE2 and its closest *Arabidopsis* homolog AtDTX12. As white lupin was reported until very recently not to be transformable (Uhde-Stone et al., 2005), we analyzed protein localization in heterologous plant species. We constructed and expressed green fluorescent protein (GFP) fusion proteins with LaMATE2 and AtDXT12. While cloning of LaMATE2-GFP was unsuccesfull, transient expression of AtDTX12-GFP in onion (*Allium cepa*) epidermal bulb cells by microprojectile delivery followed by confocal laser-scanning microscopy resulted in a weak green fluorescence at the plasma membrane (Figure 3A). In addition to AtDTX12-GFP, empty pGFP2 vector resulting in the expression of soluble GFP and a construct carrying KCO1-pEGFP-N1,

a vacuolar membrane protein (Czempinski et al., 2002), were transiently transformed in the onion epidermal cells by particle bombardment. Soluble GFP exhibited fluorescence in the nucleus and in the cytoplasm (Figure 3B), while KCO1-GFP was present as single fluorescent line sourronding the large central vacuole (Figure 3C), providing further evidence for DTX12 plasma membrane localization. LaMATE1 has been previously reported to be localized at the plasma membrane (Uhde-Stone et al., 2005).

Both the high expression level in the mature cluster roots and the localization at the plasma membrane of proteid root cells, lead us to hypotesize that the LaMATE1 transporter could function as efflux carrier for citrate or other low molecular weight compounds. Direct and indirect evidence for LaMATE2 suggest a similar function for this protein.

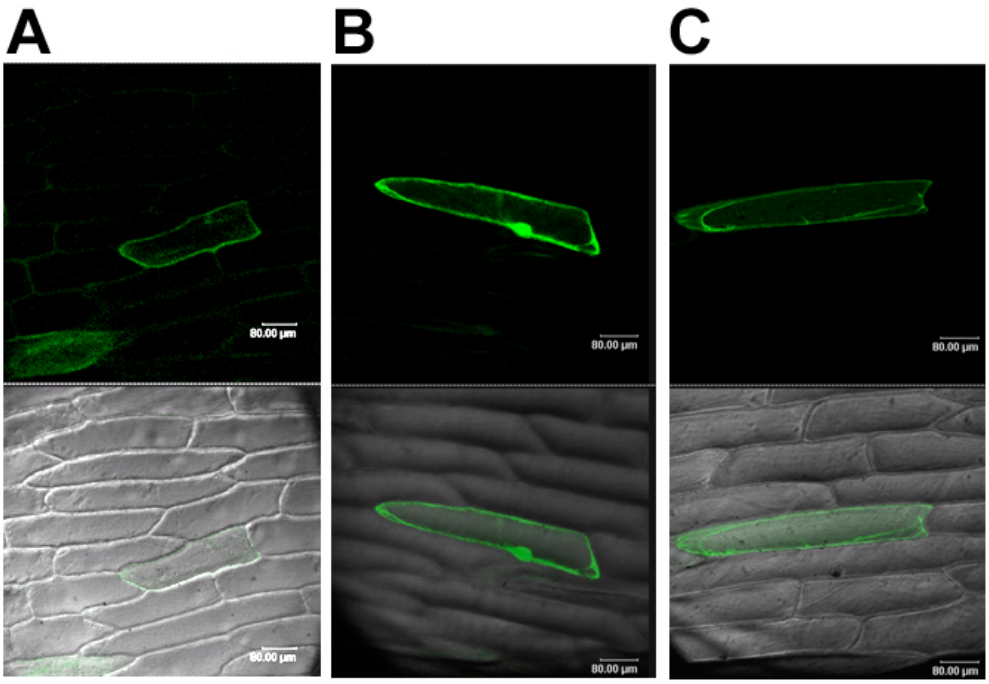

Figure 3. Localization of AtDTX12 protein in epidermal onion (*Allium cepa*) cells. Subcellular localization of AtDXT12 **(A)** by transient expression of GFP- AtDXT12 fusion protein in onion epidermal cells. As control, subcellular localization analysis of the empty vector pGFP2 **(B)** and the tonoplast protein KCO1 **(C)** are also shown.

Isolation of a lupin glycosyltransferase

Glycosylation of endogenous secondary plant products increases their water solubility, thereby reduces their potential toxicity, and enables either vacuolar deposition or efflux in the apoplast. The substrates of the transporters at the tonoplast or at the plasmamembrane are normally conjugated compounds. The enzymes involved in

transferring sugars from a range of nucleoside donors onto acceptor molecules are known as glycosyltransferases (GTs) (Jones and Vogt, 2001; Li et al., 2001; Paquette et al., 2003). GT enzymes normally utilize small molecular weight compounds as acceptor substrates and UDP-sugars as donors, catalyzing for example the glycosylation of aglycone flavonoids or other secondary metabolites. To investigate LaMATE transport properties, by their functional expression in a heterologous system, we generated a cDNA library from juvenile cluster roots of white lupin with the aim of isolating clones corresponding to a glycosyltransferase. The screening of the cDNA library resulted in the isolation of a positive clone corresponding to a putative glycosyltransferase sequence, as deduced from its homology to a putative glycosyltransferase from *Arabidopsis thaliana* (At3g28180) (Figure 4). Unfortunately, when we sequenced and analyzed the cDNA clone, we found that 464 bp in the 5' region were missing. Since inverse PCR approaches failed to amplify the region of interest, we decided to perfom a screening of a lupin genomic library. To date, three clones containing a glycosyltransferase sequence have been isolated.

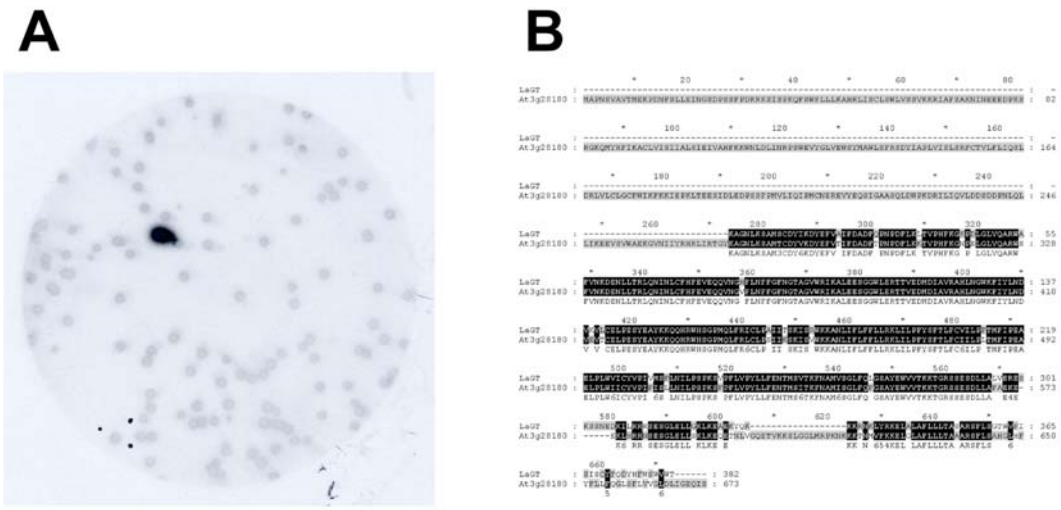

Figure 4. Isolation of a positive clone of *LaGT* from a white lupin juvenile cluster root cDNA library. (A) Autoradiography of the filter containing the positive clone of *LaGT*, hybridized with a *LaGT* probe. **(B)** Alignment of LaGT and At3g28128 protein sequences.

Discussion and outlook

In white lupin cluster roots, the exudation of organic acids and isoflavonoids in response to phosphorus deficiency has been reported and their role in the physiology of P acquisition partially clarified. Although several studies focused on the elucidation of

citrate and malate exudation mechanisms, so far nothing is known about the transporters involved in isoflavonoid secretion in the rhizosphere of white lupin. The preliminary data we collected in this work supports the hypothesis that proteins belonging to the family of MATE transporters are candidate transporters which may be involved in the exudation of citrate and/or isoflavonoids from white lupin cluster roots. We have shown that *LaMATE1* and *LaMATE2* are specifically induced upon phosphate starvation, with the highest accumulation of mRNA transcripts in the mature cluster roots, where solubilization, soil extraction and uptake of phosphate into the plant occur. The localization of the closest *Arabidopsis* homolog (AtDXT12) to LaMATE2 and LaMATE1 at the plasma membrane, further supports the idea of MATE transporters as candidates for citrate and/or isoflavonoid transporters. However, due to the technical problems encountered during the molecular work and because white lupin was reported until very recently not to be transformable (Uhde-Stone et al., 2005), the evidence so far available allows only for speculation. More specifically, the biochemical characterisation of transpor activities is missing.

As an outlook, it is intended to elucidate the transport functions of the two lupin MATE transporters and characterize biochemically the citrate and isoflavonoid efflux systems present in cluster roots of Pi-starved lupins. In the following two paragraphs I will describe experiments we have planned to use for this purpose. They represent part of a grant proposal that has been submitted in collaboration with Dr. Markus Klein.

Biochemical characterisation of plasma-membrane carboxylate and isoflavonoid transport systems

We will study the biochemical properties of plasma-membrane efflux systems for citrate/malate and genistein using plasma-membrane vesicles from lupin roots, more specifically vesicles isolated from Pi-supplied roots, Pi-starved apices and cluster roots. Based on the metabolic characterisation of exudation processes, we hypothesize that carboxylate and isoflavonoid transport activities are maximal in plasma-membrane vesicles isolated from cluster roots of lupin plants grown under phosphate stress.

As part of the cluster root project, we have successfully established the isolation of plasma membrane vesicles from different lupin root tissues (Tomasi et al., in preparation). Furthermore, our laboratory has the technical knowledge and all tools to isolate transport-competent, sealed inside-out plasma membrane vesicles of high purity

from different sources obtained by partitioning in aqueous polymer two-phase systems (Palmgren et al., 1990).

Radioactive transport substrates, namely [^{14}C]-citrate, [^{14}C]-malate and [^{3}H]-genistein, have already been obtained from commercial sources. The inside-out oriented membrane vesicles will be used to study the flux of radioactive tracers from the medium (corresponding to the cytoplasm) into the vesicular lumen (corresponding to the apoplastic space). Technically, we will use the well-established rapid filtration technique to follow the carboxylate and isoflavonoid vesicle transport. We will study the energization of the transport systems by external addition of MgATP. In case MgATP stimulates the vesicular accumulation of carboxylates or genistein, either a directly energized transport mechanism – most probably an ABC-type transporter – or a secondary energized efflux system has to be proposed. In the latter case, the proton gradient or the membrane potential difference established by the H^+-ATPase should provide the energy for substrate. Using inhibitors, most importantly uncouplers either dissipating the pH gradient or the membrane potential difference across the plasma membrane, it will be possible to distinguish between potential- or H^+-antiport-driven carboxylate and isoflavonoid efflux. In contrast, an ABC transporter-mediated efflux is expected to be insensitive to uncouplers and sensitive to vanadate. In order to differentiate between simple diffusion and carrier-mediated transport, saturation kinetic experiments will be performed and competition experiments with structural analogs of citrate and flavonoids will allow the determination of substrate specificities.

With respect to the carboxylate efflux studies we expect to obtain biochemical data that will allow either confirmation of the existence of a membrane potential-driven citrate 'channel' such as the lupin IRAC (Zhang et al., 2004) or provide further evidence for the existence of carboxylate efflux systems in addition to the IRAC conductance which does not correlate with citrate efflux activities in cluster roots.

Biochemical characterisation of transport processes catalyzed by the two lupin MATEs.

The homology to known MATE transport systems in *Arabidopsis*, namely FRD3 and TT12, and the localization of MATE1 to the plasma membrane, strongly suggest that they might be responsible for the two most striking efflux processes, namely carboxylate and isoflavonoid (genistein) secretion into the rhizosphere.

Thus, we will investigate whether the two MATE transporters are able to catalyze the efflux of carboxylates (citrate and malate) and/or the isoflavonoid genistein, a major component of the flavonoids secreted from cluster roots. These experiments will be performed by heterologous expression of both lupin *MATE* genes in the yeast *Saccharomyces cerevisiae*.

We have already cloned both *MATE* genes into shuttle vectors allowing yeast expression from a 2μ plasmid. Our previous data have demonstrated that wild-type yeast as well as our collection of mutants in multidrug transport systems are not affected in growth by the presence of increasing concentrations of flavonoids such as genistein. Thus, a simple growth-assay (e.g. the classical 'drop test') will not be helpful to understand whether one of the MATEs acts as an isoflavonoid efflux system reducing the cytoplasmic concentrations of genistein. In contrast, since yeasts are able to grow on carboxylates as the sole carbon source we will analyse whether yeasts expressing a lupin MATE exhibit reduced growth on citrate- or malate-containing media because citrate imported into the yeast is secreted again by the MATE transporter reducing the amount of available carbon for growth. We will take care to perform these assays at different pH values in order to differentiate between diffusion of the uncharged carboxylates and carrier-mediated transport of the anions. Furthermore, we will use a Δ*pdr12* yeast strain for transformation since the ABC transporter PDR12 has been shown to be involved in resistance to short-chain monocarboxylates by pumping them into the media (Piper et al., 1998). In the Δ*pdr12* yeast mutant we therefore expect that the endogenous carboxylate secretion is drastically reduced which will help to evaluate the contribution of the lupin MATEs.

Finally and most importantly we will perform transport experiments using our radioactive tracers. Again, we will use vesicle isolation and the rapid filtration technique in order to analyse the transport activities of the lupin MATEs with [^{14}C]-citrate, [^{14}C]-malate and [^{3}H]-genistein. Purification of functionally sealed cytoplasmic side-out plasma membrane vesicle fraction obtained by aqueous two-phase partitioning (Menendez et al., 1995) will allow us - like for the lupin cluster roots - to study transport across the yeast plasma membrane in a known orientation. Furthermore, with regard to carboxylate transport experiments, we will reduce contaminating activity of vacuolar carboxylate transport systems. We will compare the transport activities between vesicles isolated from MATE-transformed and control-transformed yeast strains and hope to identify with this approach whether the two MATE transporters are either able to catalyze isoflavonoid or carboxylate efflux. As already mentioned, an analysis of the kinetic

mechanism of the MATE transporters will be possible using MgATP in combination with inhibitors: If - for example - one MATE acts as an isoflavonoid/H^+-antiporter utilizing the pH gradient established by the plasma membrane H^+-ATPase we would expect (i) MgATP-stimulation, (ii) sensitivity to reagents destroying pH gradients such as NH_4Cl, but (iii) insensitivity to reagents uncoupling the membrane potential. The investigation of the transport systems will again involve analysis of kinetic determinants and competition experiments.

Material and Methods

Plant material and growth conditions

White lupin plants (*Lupinus albus* L. cv. Amiga, Südwestdeutsche Saatzucht, Rastatt, Germany) were grown in hydroponic conditions in presence (+P) or absence (-P) of P source as described by Massonneau *et al.* (2001). Seeds were incubated overnight in aerated water. Then they were kept for 3 days in dark and one day in light in filter paper soaked in 0.2 mM $CaCl_2$ to allow them to germinate. Seedling were transferred to a hydroponic culture with the following medium: 0.05 Mm Fe(III)- EDTA, 2.5 mM $Ca(NO_3)_2$, 0.9 mM K_2SO_4, 0.8 mM $MgSO_4$, 38 µM H_3BO_3, 12.5 µM $MnSO_4$, 1.25 µM $CuSO_4$, 1.25 µM $ZnSO_4$, 0.33 µM $(NH_4)_6MO_7O_{24}$, 62,5 µM KCl and with 0.25 mM KH_2PO_4 in case of P-sufficient condition. Fe deficient white lupins were grown one week after sowing in +Fe conditions and then transferred to a -Fe medium for one week before harvest. Plants were all grown at 22°C and 65% relative humidity with a light period of 16 h at 200 µmol m^{-2} s^{-1}.

Harvest of different cluster root parts

The different stages of proteoid roots were harvested as described by Massonneau et al. (2001) on five week-old plants. Normal roots were separated between slow growing secondary roots placed in the bottom part of the root system (N1), the apex (1cm) of fast growing secondary roots in the upper part of the root system, holding clusters (N2). In order to differentiate the developmental stages of root clusters, the root system was immersed in a pH-indicator solution, which indicates acidification in mature cluster regions (Neumann and Romheld, 1999). Juvenile cluster roots are emerging and growing;

immature cluster roots are fully-grown and are not acidifying, they are placed between juvenile and mature cluster roots. Mature cluster roots are also fully-grown and strongly acidify their surrounding; senescent cluster roots are brownish and non-acidifying clusters placed between mature cluster roots and the primary root.
In Fe deficient plants, only apex (5mm) well separated from cluster rootlets, and the following cluster, divided in growing rootlets (juveniles) and fully-grown ones (matures),were harvested.

cDNA-AFLP

The RNAs from juvenile, mature and senescent cluster roots were compared by cDNA-AFLP technique (Bachem et al., 1996). The procedure used in this work is described by Massonneau et al. (2001).

Construction of the cDNA library from juvenile cluster roots and screening

The library was constructed from mRNA isolated from juvenile cluster roots. cDNAs synthesis and cloning were performed using the ZAP-cDNA Synthesis Kit and ZAPcDNA Gigapack III Gold Cloning Kit (Stratagene, Amsterdam). Phage plaque lifts were performed as described by the manufacturer and the screening of LaGT mRNA performed using a radioactive probe corresponding to a partial sequence of a lupin GT. The sequences of the primers used to amplify the fragment corresponding to LaGT probe are 5' GGCATTCCATTTCGAGGTGGAAC 3' (GT2s) and 5' AGAATTATGCAAAACAGGGTGAA 3' (GTpa), which amplified a 416-bp fragment that was used as a template for random priming labeling with α–^{32}P dCTP following the protocol of NEBLOT kit from BioLabs, USA. The filters were crosslinked with UV light using the autocrosslink setting on the Stratalinker UV crosslinker (GS GENE LINKER, Bio-Rad, 120.000 μJ of UV energy) for ~30s, prehybridized in 40 ml Denhardt's solution for 1h at 65° and hybridized after addition of the radioactive probe overnight in the same solution. Washing conditions were: 2X SSC, 0.1 % SDS for 20' and 1X SSC, 0.1 % SDS for 10'. The filters were exposed to an x-ray film (BioMax MS film, Kodak) for 2 days for autoradiography and developed in the dark. Positive radioactive signals corresponding to the plaques of interest were isolated and *in vivo* excised following the protocol of the

ZAP-cDNA Synthesis Kit and ZAPcDNA Gigapack III Gold Cloning Kit (Stratagene, Amsterdam). Positive clones were completely sequenced.

Construction of the lupin genomic library

The genomic library was produced by isolating DNA from young leaves of white lupin. gDNA was cut with Sau3a to produce the DNA fragments of 9 to 23kb, and load on an agarose gel. The band corresponding to 9-23kb was recovered and purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham). The purified white lupin gDNA was used to create the genomic library using the Lambda DASH®II/BamHI/Gigapack®III Kit (Stratagene) following manufacturer's instructions. Screening with the same LaGT probe used for the cDNA library screening, allowed us to isolate some phages containing the LaGT sequence. Their DNAs were extracted, digested and cloned in pBluescript SK2 (Stratagene). Once in the bacterial vector, the genomic DNAs were sequenced.

Reverse transcription(RT)-PCR

Exctraction of the total RNA from the different cluster root developmental stages and the reverse transcription were performed as described by Massonneau et al. (2001). An aliquot of 1/100 of the RT reaction product was used for each PCR reaction. The primer sequences used in the semi-quantitative RT-PCR with α^{33}P-labelled dCTP are: La01 5' AAG CCA CCC AAT GTC TGT GT 3' and La02 5' CCC CAT GAT TAC ATT GGA TAG G 3' for *LaMATE1*; La09 5' TAGGGGCCTTCTACCTCCTT 3' and La10 5' GCAAAAATGGGAATAAGCTCA 3' for *LaMATE2*. The annealing temperature was 55° C. After 24 cycles, when strict proportionality between cycles number and amplification was observed for all tissues studied, the PCR fragments were separate on agarose gel and blotted onto a nylon membrane for autoradiography. Controls with the constitutive ribosomal protein S101 were performed in the same conditions using the following primers S101fw 5' TGCTAGGTCGTCTTGCTTCA 3' and S101rev 5' GAGCAAGAGCATGTTCACCA 3'.

Subcellular localization

To localize AtDTX12, the respective cDNA was clone in frame to the N terminus of GFP into the vector pGFP2 (Haseloff and Amos, 1995). GFP fusion construct was transiently expressed in onion (*Allium cepa*) epidermal cells using a Helium Biolistic

Particle Delivery system (Bio-Rad). After 48h of incubation in the darks, fluorescent onion epidermal cells were imaged by confocal microscopy.

Protein sequence analysis

DNA sequencing was performed at the GATC-biotech center, Konstanz (Germany) and DNA sequences were analysed with DNASTAR Lasergene® software package and by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment was performed with CLUSTALW accessible on the EMBnet website at http://www.ch.embnet.org/software/ClustalW.html. Protein topology predictions were performed using the programs available at the ExPASy Proteomic Server (http://www.expasy.org/) of the Swiss Institute of Bioinformatic. Prediction of the subcellular location was perfomed using TargetP 1.1 server (http://www.cbs.dtu.dk/services/TargetP/). Hydropathy analysis and prediction of transmembrane regions were performed using TopPred (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html).

3.4 Altered profile of secondary metabolites in the root exudates of Arabidopsis ATP-Binding Cassette (ABC) transporter mutants

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Reprinted from: Plant Physiol. First published on December 7, 2007; 10.1104/pp.107.109587

In collaboration with the group of Professor Vivanco from the Colorado State University, we compared by an HPLC-MS approach the root exudation profiles of seven ABC transporter mutants with those of the wild type, and found that three non-polar phytochemicals were missing in the root exudates of the various mutants. All tested mutants were deficient in either one or two compounds, and the same compound was sometimes missing in more than one mutant, indicating that more than one ABC transporter can be involved in the secretion of a given phytochemical or that a single transporter can be involved in the secretion of more than one secondary compound. The HPLC eluant corresponding to the peak area of one of the three missing compounds was collected and further carachterized by QTFO-MS/MS and UV-HPLC. Additionally, the metabolites present in the root exudation of the mutants were analysed by GS-MS, which allowed for the identification of groups of compounds differentially accumulating in some of the mutants compared to the wild type.

This work provides for the first time direct evidences that different members of the ABC transporters family are required for the exudation of phytochemicals from the roots.

Altered Profile of Secondary Metabolites in the Root Exudates of Arabidopsis ATP-Binding Cassette Transporter Mutants[1][C][W][OA]

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Following recent indirect evidence suggesting a role for ATP-binding cassette (ABC) transporters in root exudation of phytochemicals, we identified 25 ABC transporter genes highly expressed in the root cells most likely to be involved in secretion processes. Of these 25 genes, we also selected six full-length ABC transporters and a half-size transporter for in-depth molecular and biochemical analyses. We compared the exuded root phytochemical profiles of these seven ABC transporter mutants to those of the wild type. There were three nonpolar phytochemicals missing in various ABC transporter mutants compared to the wild type when the samples were analyzed by high-performance liquid chromatography-mass spectrometry. These data suggest that more than one ABC transporter can be involved in the secretion of a given phytochemical and that a transporter can be involved in the secretion of more than one secondary metabolite. The primary and secondary metabolites present in the root exudates of the mutants were also analyzed by gas chromatography-mass spectrometry, which allowed for the identification of groups of compounds differentially found in some of the mutants compared to the wild type. For instance, the mutant *Atpdr6* secreted a lower level of organic acids and *Atmrp2* secreted a higher level of amino acids as compared to the wild type. We conclude that the release of phytochemicals by roots is partially controlled by ABC transporters.

The primary functions of roots are the physical anchoring of plants in the soil and the absorption of nutrients and water. Another important function that has attracted much recent attention is the secretion of active biochemicals by the roots. Root secretions, or root exudates, help roots penetrate the soil (Iijima et al., 2003) and orchestrate rhizosphere interactions, including symbiotic, pathogenic, and allelopathic interactions. Hence, exudates play a central role in plant growth in natural habitats (Colebatch et al., 2002; Walker et al., 2003; Bais et al., 2004). Root exudates are composed of both low and high M_r components, including an array of primary and secondary metabolites, proteins, and peptides (Bais et al., 2006; Weisskopf et al., 2006). The compounds secreted vary in quantity and chemical structure depending on the plant species, the developmental stage, the interacting organism(s), and a wide variety of environmental factors (Bais et al., 2006). Knowledge of specific genes and mechanisms involved in root secretions remains scant. A plasma membrane H^+-ATPase and anion channels were proposed in wheat, lupine, and soybean to account for the root secretion of malate, citrate, and oxalic acid, respectively (Neumann et al., 1999; Yan et al., 2002; Sasaki et al., 2004; Shen et al., 2005). Recently, Loyola-Vargas et al. (2007) and Sugiyama et al. (2007) used a pharmacological approach to provide evidence that root secretion of certain secondary metabolites is an ATP-dependent process and suggested that ATP-binding cassette (ABC) proteins, among other transporters, could be implicated in this process.

The ABC transporters encompass a large protein family found in all phyla and utilize the energy of ATP hydrolysis to translocate solutes across cellular membranes (Higgins, 1992). In bacteria, ABC transporters function

[1] This work was supported by the National Science Foundation (grant nos. MCB–0542642 to J.M.V. and F.R.S. and MCB0416471 to L.M.B.), and by the Swiss National Foundation within the National Centres of Competence in Research "Plant Survival" and the European Union Project Plant Transporters (grant no. HPRN–CT–2002–00269 to E.M.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.107.109587

in both import and export. In eukaryotes, until recently it was thought that ABC transporters function only in export (Saurin et al., 1999). However, recent results provide evidence that some plant ABC transporters also function as importers (Shitan et al., 2003; Santelia et al., 2005; Terasaka et al., 2005). ABC transporters play a central role in many biomedical phenomena, including genetic diseases such as cystic fibrosis, multidrug resistance in cancer patients, and human pathogenic microbes (Borst and Elferink, 2002). These transporters mediate diverse cellular transport processes, such as the excretion of potentially toxic compounds, lipid translocation, heavy metal tolerance, modulation of the activity of ion channels, and nutrient transport and salt stress (Balzi and Goffeau, 1994; Szczypka et al., 1994; Higgins, 1995; Maathuis et al., 2003). Completion of the Arabidopsis (*Arabidopsis thaliana*) sequence revealed 129 ABC transporter genes that are subdivided into 13 subfamilies (Sanchez-Fernandez et al., 2001; Martinoia et al., 2002; Garcia et al., 2004). Interestingly, the number of Arabidopsis full-size ABC transporters exceeds those reported in yeast (*Saccharomyces cerevisiae*; Decottignies and Goffeau, 1997) and in humans (Dean et al., 2001), reflecting that the sessile life of plants requires many adaptive strategies and suggests that the higher number of potential substrates (secondary metabolites) produced by plants compared to other organisms may need a higher number of transporters (Dixon, 2001).

In plants, the best-characterized ABC transporters are the full-size subfamilies of transporters, including the multi-drug resistance-related protein (MRP) transporter subfamily, the pleiotropic drug resistance protein (PDR) subfamily, and the multi-drug resistance P-glycoproteins (PGP) subfamily (Martinoia et al., 2002; Crouzet et al., 2006; Geisler and Murphy, 2006; Klein et al., 2006). The MRP transporters are known to function in vacuolar sequestration of glutathionylated compounds, malonylated chlorophyll catabolites and glucuronides (Theodoulou, 2000; Martinoia et al., 2002; Rea, 2007), and in guard cell ion flux (Klein et al., 2003). PDR subfamily members are characterized in fungal systems as efflux transporters of cytotoxic compounds (Balzi and Goffeau, 1994) and in plants they are involved in exporting antifungal diterpene defense compounds to the leaf surface (Jasinski et al., 2001; Stukkens et al., 2005) and also in disease resistance (Kobae et al., 2006; Stein et al., 2006). Members of the third subfamily, the PGP transporters, are characterized to date as being implicated in the transport of the phytohormone auxin (Noh et al., 2001; Geisler et al., 2005; Terasaka et al., 2005; Geisler and Murphy, 2006). The ATH subfamily of half-molecule transporters (16 members in Arabidopsis) is not represented in yeast and little is known about their function in plants (Martinoia et al., 2002).

Generally, the root exudation of phytochemicals is assumed to occur near the root tip and root elongation zone (Hawes et al., 1998; Bais et al., 2002). Therefore, we hypothesized that ABC transporter genes specifically expressed in the epidermis of these regions may likely be involved in the root secretion processes. Here, we have distilled a list of 25 ABC transporters specifically expressed or expressed at higher levels in the cells of the root cap (stage 1), root elongation zone (stage 2), and root hair zone (stage 3) from the data set of Birnbaum et al. (2003), an exhaustive report on the microarray expression analyses of different types of root cells (i.e. lateral root cap cells, endodermis, atrichoblasts, etc.) in Arabidopsis. We correlated this information to other studies that show organ-specific expression of ABC transporters in plants (van den Brûle and Smart, 2002; Santelia et al., 2005). Here, we present a comprehensive report on seven of these ABC transporter genes spanning four subfamilies (MRP, PDR, ATH, and PGP) that are expressed in the root cells and that are involved in the secretion of phytochemicals, employing a combined genetic and biochemical approach.

RESULTS

In Silico Expression Analyses of 25 ABC Transporter Genes Present in the Roots

After examining the microarray gene expression dataset of individual cells of Arabidopsis roots (Birnbaum et al., 2003), we identified 25 ABC transporter genes exclusively or highly expressed in the cells of the endodermis, endodermis-cortex region, atrichoblast, and lateral root cap cells at three different root stages starting from the root tip. Nine subfamilies of ABC transporter genes were found to be expressed in these root cells (Table I), including PDR, MRP, PGP, and ATH. Among these subfamilies, we did not consider the NAP and GCN subfamilies because they lack a transmembrane domain and either do not function as transporters or the corresponding transmembrane regions are not known (Sanchez-Fernandez et al., 2001). We then analyzed the tissue- and organ-specific and growth-stage-dependent expression pattern of these 25 genes using the Gene Atlas and Gene Chronologer tools of the Genevestigator Web page (Supplemental Tables S1 and S2). The genes of interest belonging to the PDR, MRP, and PGP subfamilies (*AtPDR2*, *AtPDR6*, *AtPDR8*, *AtPDR9*, *AtPDR11*, *AtMRP8*, *AtPGP1*, and *AtPGP4*) are highly expressed in the lateral root cap cells and atrichoblasts, except *AtMRP2* and *AtMRP5*, which show high expression in the cells of the endodermis (Table I). While all these genes are highly or predominantly expressed in roots, they are also expressed in other plant organs (Supplemental Table S1; van den Brûle and Smart, 2002; Santelia et al., 2005). Furthermore, growth-stage-specific expression patterns indicate high transcript abundance in several of the 25 genes (Supplemental Table S2) at days 14 to 17 of plant age, except *AtPDR2*, *AtPDR8*, *AtPDR9*, *AtMRP5*, *AtMRP8*, and *AtPGP1* in the PDR, MRP, and PGP subfamilies. The expression analysis of these 25 ABC transporter genes throughout Arabidopsis growth reveals three characteristic patterns of expression. Most genes show maximum expression at 14 to 17 d after germination (subfamilies PDR, MRP,

Table I. *Array values of 25 ABC transporter genes expressed in the cell types of Arabidopsis root*
Depicted are the signal intensity values of array analyses. LRC, Lateral root cap.

Gene ID	Pump Type	Cell Expression Values						
		Stage 1	Stage 2	Stage 3	Endodermis	Endo-Cortex	Atrichoblast	LRC
At5g39040	TAP2	452.1	333.7	228.3	223.8	73	234.3	231.8
At4g15230	PDR2	716.9	672.2	606.6	160.4	143.9	656.5	563.8
At2g36380	PDR6	1,656.9	2,124.4	3790	2,647.2	1,290.4	2,305.1	2,568.3
At1g15210	PDR7	1,220.4	2,349.3	3,853.2	3,443.8	1,762.6	1,655.2	1,862.1
At1g59870	PDR8	481.9	323.6	1,446.8	3,920.8	2,384.3	1,548.4	6,630.3
At1g15520	PDR9	62.8	82.9	70.1	1,526.7	320	664.9	3,895.4
At3g53480	PDR11	2,238.7	2,524.9	2,685.1	810	542.2	2,611.3	5,093.3
At2g34660	MRP2	648.9	991.1	1,452	1,117.1	551.4	461.9	669.5
At1g04120	MRP5	368.5	744.5	710	696.6	472.6	395.9	395.5
At3g21250	MRP8	318.3	760.9	532.4	257.4	154.4	506.5	387.7
At2g39350	WBC1	585.2	320.6	154	1,049.4	351.6	2,202.3	4,087.8
At2g28070	WBC3	755.5	435.1	211.5	209.9	339.5	298.3	282.4
At4g04770	NAP1	635.8	568.4	750.1	493.2	323.2	326.6	253.7
At1g67940	NAP3	185.6	296.8	237.8	123.8	211.3	350.9	334.7
At1g71330	NAP5	230.1	190.6	164.2	154.8	192.5	239.6	281
At3g10670	NAP7	277.8	247.7	198.5	292.4	237.8	244	233.6
At5g02270	NAP9	857.5	2,912.4	3,597.8	1,208.8	697.7	623.6	527.1
At3g47730	ATH1	603.1	383.5	1,075	606.1	349.6	420.5	1,507.7
At3g47780	ATH6	451.5	331.1	586.3	247.7	150.6	315	578
At4g01660	ATH10	1,009.6	1,321.8	981.2	593	360.3	496	358.9
At5g58270	ATM1	605.2	484.5	420.4	421.1	253.7	294.7	237.2
At5g60790	GCN1	1,547.3	1,086.8	699	991.2	2,409.6	2,022.7	2,080.2
At1g64550	GCN3	1,707.6	1,236.5	583.9	572.7	567.4	723.3	504.6
At2g36910	Pgp1	728.2	1,280.6	1,299.2	1,023.4	831.2	1,121.3	274.3
At2g47000	Pgp4	447.6	1,005	2,106.9	1,140.1	580.3	700.6	787.7

WBC, NAP, and PGP). Some genes, such as *AtTAP2*, *AtMRP5*, *AtNAP5*, *AtATH1*, *AtATM3*, and *AtGCN1*, show high transcript abundance in the last stage of development (45–50 d). Genes *AtMRP8* and *AtPGP1* are highly expressed in the preceding stage (36–44 d). Among these 25 ABC transporter genes, we selected seven genes comprising full-length (*AtPGP1*, *AtPGP4*, *AtPDR2*, *AtPDR6*, *AtPDR7*, and *AtMRP2*) and half-size (*AtATH6*) transporters for detailed genetic and biochemical studies.

Phenotypic Characterization of ABC Transporter T-DNA Knockout Mutants

As detailed in the experimental procedures, the ABC transporter T-DNA knockout (KO) mutants corresponding to the six full-length ABC transporter genes and one half-size transporter were first screened to select homozygous individuals; we then identified the T-DNA insertion region in the gene. Subsequently, RT-PCR assays were conducted to check for complete KO of the gene, and the mutants were screened for visible phenotypes. The T-DNA deletion mutants for the four genes coding for the ABC transporters AtPGP1, AtATH6, AtMRP2, and AtPDR7 were found on the ninth, 12th, 11th, and fifth exons, respectively, in their gene, and the T-DNA insertions for the mutants *Atpdr2* and *Atpdr6* were found on the 21st intron and the third intron, respectively. All seven ABC transporter mutants show complete KO of the gene, except *Atpgp4-2*, which has an insertion 133 bp downstream of the stop codon and therefore is able to create a full-length transcript. This transcript appears as a faint band, indicating that RNA stability is affected, probably resulting in a leaky mutant (Fig. 1). Root phenotype analysis showed that the *Atmrp2* mutant has significant primary root length reduction (data not shown) and that *Atpgp1* has a significant increase in the amount of lateral root formation compared with the wild type. Besides this root phenotype, there is no observable phenotype difference in terms of shoot morphology or bolting or flowering time between the mutants and the wild type under the conditions tested.

HPLC-Mass Spectrometry Analysis of Root Secretion and Root Tissue Metabolite Profiles for the Selected PDR, PGP, ATH, and MRP Arabidopsis Mutants

To explore the role of the seven ABC transporters in the root exudation of phytochemicals, the root exudation profiles of the mutants were analyzed by HPLC-mass spectrometry (MS) and compared with the wild-type profiles at two different time points: 3 and 7 d after continuous secretion of phytochemicals, corresponding to 21- and 25-d-old plants, respectively (see "Materials and Methods"). Based on the extraction method, we found three nonpolar phytochemicals missing in the root exudates of the various mutants compared to those of the wild type after 3 d of continuous root exudation (Table II; Supplemental Fig. S1). Compound 1,

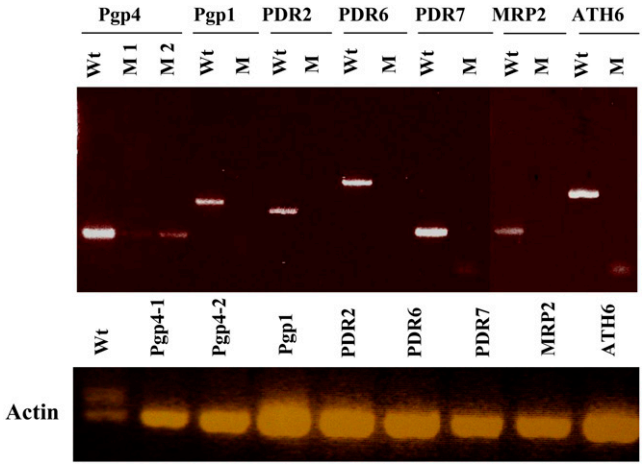


Figure 1. RT-PCR assays of the ABC transporter mutants used in this study. cDNA was prepared from 1.5 μg of root tissue total RNA with superscript reverse transcriptase (Invitrogen) and RT-PCR was performed using gene-specific primers. Below is the actin control for the cDNA of mutants and the wild type. The corresponding Salk line mutants for these ABC transporters are listed in Supplemental Table S3. The primers used for this RT-PCR are listed in Supplemental Table S5. Wt, Wild type; M, mutant lines. [See online article for color version of this figure.]

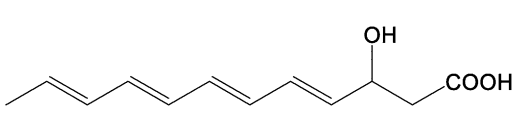
with a retention time of 33.92 min and a M_r of 208, was absent in the exudates of the mutants *Atpdr6*, *Atpdr7*, and *Atath6*. Compounds 2 and 3, with retention times of 38.9 and 39.9 min and M_r of 387 and 647, respectively, were absent in the exudates of the mutants *Atmrp2* and *Atpgp1*. Similarly, compound 3 was also absent in the exudates of the mutants *Atpgp4* and *Atpdr2*. We examined the root exudates in the mutants and wild-type plants for the same three compounds after 7 d of continuous root exudation (Table III; Supplemental Fig. S2). The results were similar to the profiles after 3 d of exudation, except that compounds 1 and 3 were absent in the exudates of the mutants *Atpdr7* and *Atpdr2* after 3 d of exudation but were present after 7 d of exudation. It is worth noting that these three compounds are present in the wild-type root exudates at both time points.

Additional studies were pursued to validate our data that ABC transporters were involved in root secretion of compounds. First, we performed complementation analyses for one ABC transporter mutant, *Atpgp4*, by using an overexpresser line in the homozygote *Atpgp4-1* mutant background (*Atpgp4OX-pgp4-1*) to test if the compound missing in the root exudates of the mutants reappears in the overexpresser line. Our results show that compound 3 with M_r 647, missing in the exudates of the *Atpgp4-1* mutant, did reappear in the overexpresser line *Atpgp4OX-pgp4-1* after 3 d of secretion of root exudates (Fig. 2), indicating that this compound could be a substrate for this transporter. It is noted that compound 3 accumulates to higher levels in the wild type compared to the *Atpgp4OX-pgp4-1*. It is possible that the transporter is only expressed in the plasma membrane in the wild type, but that in the overexpresser line the transporter is expressed throughout the plant, with the compounds normally secreted from the root being secreted at other locations, resulting in lower levels of this compound in the root exudates. Second, to prove that root-expressed ABC transporters are involved in the root exudation process, we examined the 3-d root exudate profile of the *Atpdr4* mutant, because even though it is highly expressed in the inflorescence of the wild type, it is not expressed in the roots (van den Brûle and Smart, 2002). This mutant showed no difference in its exudate profile compared to the wild type (Supplemental Fig. S3).

The root tissue phytochemical profiles were further analyzed in all seven mutants at the same time points as those used for the root exudates, and those profiles were compared with those of the wild-type roots. None of the three affected root-secreted compounds were observed in the mutants or wild type, but there were significant differences in the phytochemicals present in the root tissues of the wild type and mutants. For example, compound 4, with a retention time of 19.3 min and a M_r of 398, was present only in the root tissue of mutants *Atpgp4*, *Atpdr6*, and *Atath6* and was not present in that of any other mutants or in wild-type 21-d-old plants (Table IV; Supplemental Fig. S4), but this compound was absent in 25-d-old plants of all mutants and the wild type (Table V; Supplemental Fig. S5). Similarly, compounds 5, 6, and 7, with retention times of 32.0, 45.11, and 46.6 min and M_r of 186, 314, and 316, respectively, were present in high concentrations in the root tissue of mutants *Atpdr2*, *Atpdr7*, and *Atmrp2*

Table II. *Retention times and M_r of the compounds missing in the ABC transporter mutants compared with wild type after 3 d secretion (21-d-old plants) of root exudate profiles analyzed by HPLC-MS*

Structure of compound 1 (3-hydroxy-4(*Z*), 6(*Z*), 8(*Z*), 10(*Z*)-tetraenoic acid) based on tentative identification by employing UV spectrum and QTOF-MS/MS. +, Presence of compound; −, absence of compound.



Peak Retention Time (M_r)	Col-0	*pgp4-1*	*pgp4-2*	*pdr2*	*pdr6*	*pdr7*	*mrp2*	*ath6*	*pgp1*
Compound 1: 33.92 min (208)	+	+	+	+	−	−	+	−	+
Compound 2: 38.9 min (387)	+	+	+	+	+	+	−	+	−
Compound 3: 39.9 min (647)	+	−	−	−	+	+	−	+	−

Table III. *Retention times and M_r of the compounds missing in the ABC transporter mutants compared with wild type after 7 d secretion (25-d-old plants) of root exudate profiles analyzed by HPLC-MS*

+, Presence of compound; −, absence of compound.

Peak Retention Time (M_r)	Col-0	*pgp4-1*	*pgp4-2*	*pdr2*	*pdr6*	*pdr7*	*mrp2*	*ath6*	*pgp1*
Compound 1: 33.92 min (208)	+	+	+	+	−	+	+	−	+
Compound 2: 38.9 min (387)	+	+	+	+	+	+	−	+	−
Compound 3: 39.9 min (647)	+	−	−	+	+	+	−	+	−

compared with that of wild-type 21-d-old plants. However, the same compounds were present in the root tissue in concentrations similar to those in the wild type in 25-d-old plants except in *Atmrp2*, which shows higher concentrations of compounds 6 and 7. In 21-d-old plants of *Atpgp4*, *Atpdr6*, and *Atath6* mutants, compounds 6 and 7 were similar in concentration in both the exudates of the mutants and the wild type; however, the same compounds were significantly higher in the mutants compared to the wild type in 25-d-old plants. Similarly, in exudates of 21-d-old plants of *Atpgp4*, *Atpdr6*, and *Atath6* mutants, compound 5 showed a significantly lower concentration compared with its presence in the wild-type exudates: however, the same compound 5 was significantly higher in the exudates of the mutants compared to those of the wild type in 25-d-old plants. In addition, the phytochemical profiles from the root exudates and root tissue of wild-type plants (21 d old) were compared, and these profiles showed at least 80% difference in their profiles (Supplemental Fig. S6).

Partial Characterization of the Missing Compound 1 with M_r 208

To identify the missing compounds in the exudates of the mutants, we collected the HPLC eluant corresponding to the peak areas showing the M_r corresponding to the compounds 1, 2, and 3 by using HPLC under the conditions described in "Materials and Methods." Sufficient material was collected for compound 1 to allow further chemical characterization. Accurate M_r measurement and tandem MS were performed using hybrid quadrupole time-of-flight tandem MS (QTOF-MS/MS). UV spectral characterization was performed using HPLC coupled with a photodiode array detector (DAD). The HPLC-DAD-detected spectrum showed absorption maximums at 240 nm and 300 nm; the relatively more intense absorption was observed at 300 nm (Supplemental Fig. S7). This type of spectrum can be attributed to a system of four conjugated trans double bonds (Scott, 1964). In addition, a high-resolution MS/MS spectrum in the positive-ion mode showed a molecular ion at 209.1159 (9%: calculated 209.1178 for $C_{12}H_{17}O_3$) and fragment ions at 163.1105 (30%: calculated 163.1123 for $C_{11}H_{15}O$), 149.0968 (90%: calculated 149.0967 for $C_{10}H_{13}O$), and 131.0861 (100%: calculated 131.0861 for $C_{10}H_{11}$; Supplemental Fig. S7). Based on the fragmentation pattern and UV spectrum data, the following structure is proposed for compound 1: 3-hydroxy-4(Z), 6(Z), 8(Z), 10(Z)-tetraenoic acid (Table II); however, additional proof through NMR analysis is needed to confirm the proposed structure.

Gas Chromatography-MS Analysis of Root Exudate Profiles for the Selected PDR, PGP, ATH, and MRP Arabidopsis Mutants

The primary and secondary metabolites present in the root exudation profiles of the mutants were analyzed by gas chromatography (GC)-MS and compared with the root exudate profiles of the wild type after 3 d of continuous root secretion of phytochemicals corresponding to 21-d-old plants (see "Materials and Methods"). A GC-MS analysis was carried out with the root exudates of the wild type and five mutants (*Atpdr2*, *Atpdr6*, *Atmrp2*, *Atath6*, and *Atpgp4*). Principal component analyses (PCAs) showed that the root exudate profiles of mutants *Atpdr6* and *Atmrp2* were clustered separately from other mutants and the wild type (Fig. 3). The identified compounds contributing most to factor 1 in PCA are urea, Tyr, and Asp. Similarly, the identified compounds contributing most to factor 2 are ethanolamine, glycolic acid, and 3-hydroxypropionic acid. On the whole, the exudate profiles of the mutant *Atpdr6* showed significantly lower levels of organic acids (3-hydroxypropionic acid, succinic acid, fumaric

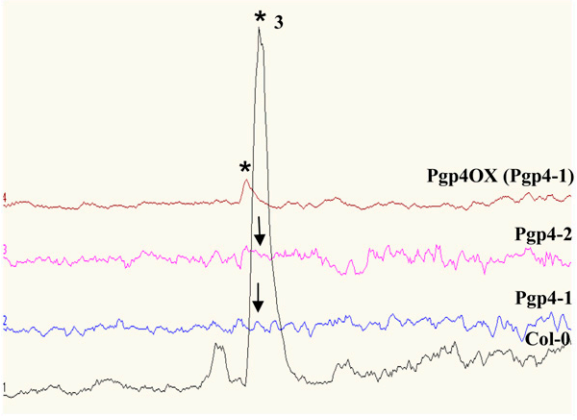


Figure 2. Profile shows the M_r trace of compound 3 (647) in the root exudates of the wild type (Col-0), ABC transporter mutant *Atpgp4-1* (*pgp4-1*), and AtPGP4 overexpresser line in the homozygote *Atpgp4-1* mutant background (*Atpgp4OX-pgp4-1*) after 3 d of continuous root secretion (details in "Materials and Methods"). Arrows indicate the absence of peak. Asterisks indicate the presence of peak. The number indicates the peak number. The results represent experiments repeated two times with three replicates each. [See online article for color version of this figure.]

Table IV. *Retention times and M_r of the compounds of the root tissue profiles of 21-day-old wild type and ABC transporter mutants analyzed by HPLC-MS*

Values represented are peak areas of the corresponding compounds in the wild type and mutants. The values in bold are statistically significant when compared to the wild type at $P \leq 0.05$, n = 6. +, Presence of compound; −, absence of compound.

Peak Retention Time (M_r)	Col-0	*pgp4-1*	*pgp4-2*	*pdr2*	*pdr6*	*pdr7*	*mrp2*	*ath6*	*pgp1*
Compound 4: 19.3 min (398)	−	+	+	−	+	−	−	+	−
Compound 5: 32.0 min (186)	37.3 ± 1.5	**12.9 ± 1.3**	**20.4 ± 2.3**	**78.9 ± 3.0**	**13.3 ± 2.0**	**62.4 ± 1.8**	**80.8 ± 1.5**	**16.7 ± 1.0**	**71.3 ± 1.2**
Compound 6: 45.11 min (314)	4.7 ± 2.0	5.3 ± 1.0	4.7 ± 1.0	**9.9 ± 1.5**	3.8 ± 2.0	**9.9 ± 1.5**	**10.2 ± 1.2**	3.3 ± 2.1	**8.1 ± 1.0**
Compound 7: 46.6 min (316)	5.0 ± 1.4	3.8 ± 0.8	3.5 ± 1.5	**8.0 ± 2.5**	2.7 ± 2.5	**8.0 ± 1.8**	**9.3 ± 1.3**	2.8 ± 2.2	5.7 ± 0.8

acid, malic acid, trihydroxybutyric acid, ribonic acid, etc.), while the *Atmrp2* mutant showed a significantly high level of amino acids (L-Ser, L-Pro, L-Ile, L-Thr, L-Gly, L-Phe, etc.) compared to the wild-type root exudate profiles (Supplemental Fig. S8). Besides these, there were significant differences in the "unknown" compounds in the exudates of the mutants compared to those of the wild type.

DISCUSSION

Generally, in living systems, molecules are transported against a concentration gradient requiring the use of energy. For several transport systems, this energy is directly provided by ATP hydrolysis. Well-known examples of ATP-powered systems are P-type ATPases and ABC transporters. It has been demonstrated that ABC transporters are involved in the membrane transport of endogenous secondary metabolites in plants (Yazaki, 2005). However, until now, only indirect evidence has been presented in support of the involvement of ABC transporters in the release of phytochemicals into the soil (Loyola-Vargas et al., 2007; Sugiyama et al., 2007). No direct genetic evidence that ABC transporters do indeed play a role in root exudation of secondary metabolites has been found. Here, we present direct evidence that ABC-type transporters are involved in the release of secondary compounds from the roots.

A recent report (Birnbaum et al., 2003) provides evidence that ABC transporters are expressed in root cells, including those in the lateral root cap, the epidermis, and the endodermis in the root cap, and the root elongation and root hair zones. We hypothesized that these ABC transporters may play a role in the root exudation process. Among the seven ABC transporters selected for this study, AtPGP1 and AtPGP4 have been shown to be localized to the plasma membrane of mature root epidermal cells, and both are involved in auxin transport (Santelia et al., 2005; Terasaka et al., 2005; Geisler and Murphy, 2006). AtMRP2 has been demonstrated to reside in the vacuolar membrane and to transport glutathione *S*-conjugates and chlorophyll catabolites (Lu et al., 1998). There are no reports available on the subcellular localization of the other transporters examined in this study (AtPDR2, AtPDR6, AtPDR7, and AtATH6), and only a few reports describe the function of plant PDRs. In these reports, it was proposed that the compounds transported by PDRs are required for plant protection against pathogen attack (Jasinski et al., 2001; Stukkens et al., 2005; Kobae et al., 2006; Stein et al., 2006). Therefore, this subfamily of ABC transporters appears to be particularly important in the root exudation of phytochemicals, and this exudation might influence the microbial composition of the rhizosphere.

HPLC-MS analyses of the seven ABC transporter mutants revealed that the mutant exudates were deficient in either one or two compounds compared with the wild-type profiles (Table II; Supplemental Fig. S1). For example, compound 1, tentatively identified as 3-hydroxy-4(*Z*),6(*Z*),8(*Z*),10(*Z*)-tetraenoic acid, is lacking in the root exudates of mutants *Atpdr6*, *Atpdr7*, and *Atath6*, indicating that one compound may act as a substrate for more than one ABC transporter. In contrast, compounds 2 and 3 were absent in the exudates of mutants *Atmrp2* and *Atpgp1*, suggesting that one ABC transporter can transport structurally and functionally unrelated compounds. Previous studies indicate that AtMRP2 and AtMRP3 can simultaneously transport endogenous substrates such as chlorophyll catabolites and glutathione compounds and that neither transport interferes with the other (Lu et al., 1998; Tommasini et al., 1998). ABC transporters can also transport a

Table V. *Retention times and M_r of the compounds of the root tissue profiles of 25-d-old wild type and ABC transporter mutants analyzed by HPLC-MS*

Values represented are peak areas of the corresponding compounds in the wild type and mutants. The values in bold are statistically significant when compared to the wild type at $P \leq 0.05$, $n = 6$. −, Absence of compound.

Peak Retention Time (M_r)	Col-0	*pgp4-1*	*pgp4-2*	*pdr2*	*pdr6*	*pdr7*	*mrp2*	*ath6*	*pgp1*
Compound 4: 19.3 min (398)	−	−	−	−	−	−	−	−	−
Compound 5: 32.0 min (186)	12.9 ± 1.0	**37.3 ± 1.8**	**32.4 ± 1.5**	13.6 ± 2.5	**78.9 ± 0.5**	16.7 ± 2.0	15.5 ± 1.0	**62.4 ± 2.0**	10.9 ± 1.6
Compound 6: 45.11 min (314)	4.7 ± 1.5	**9.0 ± 2.2**	**8.3 ± 1.6**	4.7 ± 2.3	**9.9 ± 1.0**	3.8 ± 1.5	**10.2 ± 1.2**	**8.1 ± 2.2**	3.3 ± 1.6
Compound 7: 46.6 min (316)	5.0 ± 2.0	**7.5 ± 0.5**	**8.4 ± 0.5**	3.8 ± 1.3	**8.0 ± 1.5**	2.7 ± 1.0	**9.3 ± 2.1**	**8.5 ± 1.1**	2.8 ± 2.2

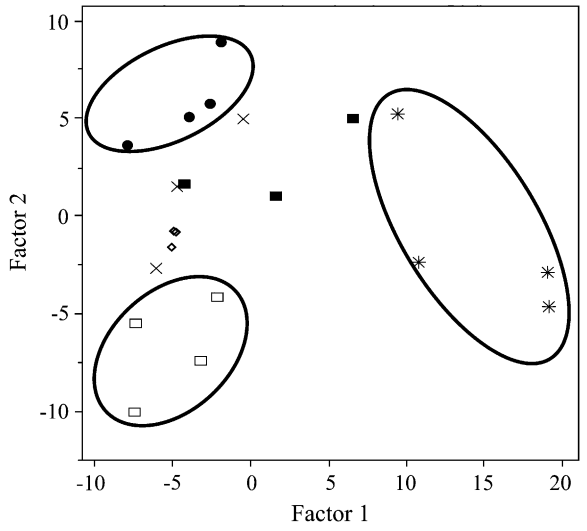


Figure 3. PCA of the root exudate profile of ABC transporter mutants compared to wild type analyzed by GC-MS. Col-0, black circles; *Atpdr6*, white rectangles; *Atpdr2*, black rectangles; *Atmrp2*, asterisks; *Atath6*, white diamonds; *Atpgp4-1*, X.

number of chemically different compounds; an example might be PDR5 from yeast, which is active in transport of numerous unrelated compounds (Kolaczkowski et al., 1996). The majority of tetraenoic fatty acids are structural monomers of the lipids present in the mitochondrial membrane, and their levels fluctuate according to the various environmental stimuli, especially low or high temperatures (Makarenko et al., 2003). Additionally, it has been reported that tetraeonic fatty acids are present in root hairs (Qu et al., 2005). We analyzed the root exudation profiles of the seven ABC transporter mutants and compared those profiles with the wild type at two time points. It was interesting to find that compounds 1 and 3 were absent in the 21-d-old plant root exudates but present in the 25-d-old plant root exudates of the mutants *Atpdr7* and *Atpdr2*, respectively (Table III; Supplemental Fig. S2). Several reasons could account for this observation, such as developmentally dependent redundancy of the ABC transporters in transporting these two compounds outside the cell.

Although only a few plant ABC transporters have been localized so far, it is known that AtPGP1 and AtPGP4 are targeted to the plasma membrane, while AtMRP2 is localized in the vacuolar membrane (Lu et al., 1998; Terasaka et al., 2005; Geisler and Murphy, 2006). Thus, we expected that the phytochemicals not detected in the mutant root exudates would accumulate inside the cell organelles. The phytochemical profile of the root exudates and root tissues of the wild-type plants were not similar and were found to have only 20% overlap based on the M_r of the compounds detected by HPLC-MS (Supplemental Fig. S6). This 80% difference between root tissue and root exudates can be explained by the fact that only a minority of the compounds synthesized in the plant are excreted. The observation that several excreted compounds are not found within the root might be due to the fact that these compounds are excreted very efficiently and that cellular concentrations are kept low. We found significant differences in the root tissue profiles of 21-d-old and 25-d-old mutants with the root tissue profiles of the corresponding wild type (Tables IV and V). Compound 4 was present in the exudates of the mutants *Atpgp4*, *Atpdr6*, and *Atath6* and absent in those of other mutants, including the wild type, suggesting that this compound may be a precursor compound and is accumulating in the vacuoles or other organelles due to the absence of the corresponding ABC transporter. AtMRP2 is localized in the vacuolar membrane and, if impaired, may affect the vacuolar transport of compounds, changing the phytochemical profile of the cytosol and the root exudation profile of the plant.

To identify other compounds (both primary and secondary metabolites) in the exudates, we analyzed the root exudates of the wild-type and ABC transporter mutants by GC-MS. Two mutants, *Atpdr6* and *Atmrp2*, show a low level of dicarboxylic acid secretion and a high level of amino acid secretion in the exudates compared to the wild type, respectively (Supplemental Fig. S8). Because we found such widespread changes in root secretion of primary and secondary metabolites in the GC-MS analysis of the ABC transporter mutants, we are tempted to suggest that all these changes are not necessarily directly due to the specific ABC transporter but rather that the lack of expression of the transporter could change the expression pattern of other transporting systems leading to changes in the secretion of several other unrelated compounds. This hypothesis is supported by the fact that in the GC-MS data, we did not detect missing compounds compared to the wild-type exudates, as observed in LC-MS analysis, but rather differences in the ratios of the same type of compounds.

It is possible that the missing compounds observed in the ABC transporter mutant root exudates are due to precursor compounds that are not translocated from shoots to roots or between organelles in the cell. ABC transporters located mainly in roots but also expressed in other tissues may be involved not only in the excretion of compounds but also in long-distance transport. In particular, the ABC transporters AtPDR2, AtPDR6, AtPDR7, and AtATH6 do not have subcellular localization data, meaning that they may indeed not be targeted to the plasma membrane and thus that intracellular transport may affect the metabolism and hence the synthesis of the compounds found in the root exudates. On the other hand, the ABC transporters AtPGP4 and AtPGP1, located at the plasma membrane mainly in the root epidermal cells, are quite likely involved in the excretion of compounds at the root level.

Taken together, these data show that ABC transporters are involved in the root exudation process. Based on the results reported here, three nonmutually

exclusive possibilities exist for how that involvement operates: one ABC transporter may transport one specific compound; one compound may act as a substrate for more than one ABC transporter; or one ABC transporter may transport a range of unrelated compounds. Thus, it could be inferred that the additional 18 ABC transporters found in Arabidopsis roots, other types of transporters (i.e. MATE), or other nontransporter mechanisms may account for the secretion of the large number of phytochemicals present in the root exudates. Current efforts were focused on identifying transport systems involved in root secretion and future efforts will focus on complete characterization of these transport systems and compounds. Elucidation of these additional secretion mechanisms will provide tools to overproduce the secretion of phytochemicals for agricultural and biotechnological benefits.

MATERIALS AND METHODS

In Silico Analyses of 25 ABC Transporter Genes

A list of 25 ABC transporter genes expressed in the root cells of Arabidopsis (*Arabidopsis thaliana*) were derived from the microarray gene expression dataset (kindly provided by Dr. Philip N. Benfey, Duke University) of individual root cells such as endodermis, endodermis-cortex region, atrichoblast, and lateral root cap cells at three different stages: stage 1, approximately 0.15 mm from the root tip (root cap); stage 2, approximately 0.30 mm from the root tip (root elongation zone); and stage 3, approximately 0.45 to 2 mm from the root tip (root hair zone; Birnbaum et al., 2003; Table I). The tissue- and organ-specific expression and growth stage-dependent expression pattern of these 25 genes were analyzed using the Gene Atlas and Gene Chronologer tools of the ''Genevestigator'' Web interface (https://www.genevestigator.eth.ch/; Zimmermann et al., 2004). The dataset was restricted to the Columbia-0 (Col-0) ecotype representing the sequenced genome and all chipsets (AtGeneExpress, NASC arrays, GEO, ArrayExpress, Gruissem Laboratory, and the Functional Genomics Centre-Zurich) were used for analysis.

Plant Material and Growth Conditions

Arabidopsis seeds were surface sterilized with bleach for 2 min followed by four rinses in sterile distilled water and plated on Murashige and Skoog (Murashige and Skoog, 1962) salts supplemented with 3% Suc and 0.7% bactoagar in petri dishes. Plates were incubated in a growth chamber (Percival Scientific) at 25°C, with a photoperiod of 16 h light/8 h dark for germination. To collect root exudates, 7-d-old seedlings were transferred to 6-well culture plates (Fischer), each containing 5 mL of liquid Murashige and Skoog (Murashige and Skoog basal salts supplemented with 1% Suc), incubated on an orbital shaker at 90 rpm, and illuminated under cool-white fluorescent light (45 μmol m^{-2} s^{-1}) with a photoperiod of 16 h light/8 h dark at 25°C. According to the methods of Loyola-Vargas et al. (2007), when plants were 18 d old, they were washed with sterile water to remove the surface-adhering exudates and transferred to new 6-well plates containing 5 mL of MS liquid media and incubated on an orbital shaker at 90 rpm and illuminated under cool-white fluorescent light (45 μmol m^{-2} s^{-1}) with a photoperiod of 16 h light/8 h dark at 25°C. The exudates were collected 3 and 7 d after transfer (plants were 21- and 25-d-old, respectively). For each replicate analysis, we collected 60 mL exudates from 12 Arabidopsis plants. For vertical plate assays, mutant and wild-type seeds were plated on Murashige and Skoog agar plates supplemented with 0.5% and 1% Suc and incubated in a growth chamber with a photoperiod of 8 h light/16 h dark and readings were taken after 7 d. The list of ABC transporter T-DNA KO mutants used in this study is provided in Supplemental Table S2.

Screening of T-DNA Mutant Homozygous Lines

Plant lines containing T-DNA insertions for the seven ABC transporter genes used in this study were obtained from the Arabidopsis Biological Resources Center and from the mutant collection of Dr. Enrico Martinoia. The T-DNA insertion and homozygote nature were verified by PCR analysis with a gene-specific primer and left border primer and further confirmed by sequencing. The complete KO of the gene product was confirmed by RT-PCR. Briefly, total RNA was isolated from root tissue using a Qiagen Plant RNAeasy mini kit, and subsequently cDNA was prepared using Superscript Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. PCR was performed by using gene-specific primers. The primers used in this study for homozygote screening and gene expression are listed in Supplemental Tables S2 and S5.

Phytochemical Extraction

Twenty-one-day-old and 25-d-old Arabidopsis seedlings grown in 6-well plates (one plant per well) containing Murashige and Skoog liquid media were used for extraction of rhizo-secreted phytochemicals. The collected liquid medium was centrifuged at 8,000 rpm for 20 min followed by filtration using Nylon syringe filters of 0.45-μm pore size (Nalgene) to remove root sheathing and root-border-like cells. After filtration, the liquid was concentrated by freeze-drying (Labconco) to remove water, and the concentrate was dissolved in 5 mL of double-distilled water. The pH was adjusted to 3.0 and the concentrate was partitioned two times with 5 mL of ethyl acetate. The ethyl acetate fractions were pooled and dried under N_2 gas. The dried concentrate was again dissolved in 100 μL of methanol and analyzed by HPLC-MS. Root tissue phytochemical extractions were carried out by grinding 50 mg of freeze-dried root tissue in 5 mL of 80% methanol and centrifuging for 20 min at 8,000 rpm to collect the supernatant before drying under N_2 gas. The dried concentrate was again dissolved in 1 mL methanol and analyzed by HPLC-MS. The experiment was repeated twice with three replicates.

HPLC and MS Analyses

The extracted phytochemicals from liquid media were chromatographed by gradient elution on a 150-mm × 4.6-mm reverse-phase, C_{18} column (Supelco). The chromatographic system (Dionex) consisted of two P680 pumps connected to an AS1-100 automated sample injector and was detected at 280 nm with a UV-visible detector. M_r determination of the peaks was performed using a quadrupole mass spectrometer detector (MSQ-MS; Thermo Electron). A gradient was used for all separations with a flow rate of 0.7 mL min^{-1}. The gradient was as follows: 0 to 10 min, 90.0% water and 10% methanol; 10 to 60 min, 10.0% to 90% (v/v) methanol and 90% to 10% (v/v) water; and 60 to 70 min, 90.0% (v/v) methanol and 10% water.

GC and MS Analyses

Root exudates for GC-MS analyses were collected in a similar manner as for HPLC-MS analyses, except that the 18-d-old plants were washed in sterile water and transferred to new 6-well culture plates containing 5 mL of sterile water instead of MS liquid media with 1% Suc. After 3 d continuous secretion, the root exudates were collected, freeze-dried, and processed following the standard methoximation and trimethylsilylation derivative procedure (Broeckling et al., 2005). One microliter of each sample was injected onto an Agilent 6890 GC coupled to a 5973 MS at a split ratio of 1:1. The initial oven temperature of 80°C was held for 2 min ramped at 5°C/min to a final temperature of 315°C and then held for 12 min. Separation was achieved using a 60 m DB-5MS (J & W Scientific; 0.25 mm i.d. and 0.25-μm film thickness) at a flow rate of 1.0 mL/min. Peak detection and deconvolution were performed with AMDIS (Halket et al., 1999) for several samples of each treatment, and peak lists were compiled in MET-IDEA (Broeckling et al., 2006). MET-IDEA was then used to extract quantitative peak area values for polar and nonpolar data. Redundant peaks were removed from the data set, peak area values were scaled to mean zero, and SD 1.0 and the resulting data matrix were analyzed with ANOVA, PCA in JMP (SAS Institute). Only P values of <0.01 were considered significant for ANOVA analyses.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Mass chromatograms of compounds 1 (208), 2 (387), and 3 (647) in the root exudates of wild-type and ABC transporter mutants for 3 d continuous root secretion of compounds (details in ''Materials and Methods'').

Supplemental Figure S2. Mass chromatograms of compounds 1 (208), 2 (387), and 3 (647) in the root exudates of wild-type and ABC transporter mutants for 7 d continuous root secretion of compounds (details in "Materials and Methods").

Supplemental Figure S3. Root exudates profile of 21-d-old wild-type (Col-0) and ABC transporter mutant *Atpdr4* (PDR4) plants analyzed by HPLC-MS at wavelength 280 nm.

Supplemental Figure S4. Metabolic profile of Arabidopsis root tissues of 21-d-old wild-type and ABC transporter mutant plants analyzed by HPLC-MS at wavelength 280 nm.

Supplemental Figure S5. Profile of Arabidopsis root tissues of 25-d-old wild-type and ABC transporter mutant plants analyzed by HPLC-MS at wavelength 280 nm.

Supplemental Figure S6. Chromatogram shows the profiles of root exudates and root tissues from wild-type plants.

Supplemental Figure S7. A, Absorption spectrum of compound 1 showing M_r 208 detected by UV coupled with DAD; and B, accurate mass and fragmentation pattern of compound 1 detected using a hybrid QTOF-MS/MS.

Supplemental Figure S8. Graphs illustrating the representative compounds that show different levels in the ABC transporter mutants compared to wild type analyzed by GC-MS.

Supplemental Table S1. Tissue- and organ-specific expression of 25 ABC transporter genes expressed in Arabidopsis roots.

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Supplemental Table S5. List of primers used in this study for RT-PCR assays.

ACKNOWLEDGMENTS

We thank Emily Wortman-Wunder for editorial assistance. We acknowledge Dr. Philip Benfey (Duke University) for providing the microarray dataset of root cells. V.M.L.-V. is recipient of a scholarship from CONACYT, Mexico.

Received September 23, 2007; accepted November 27, 2007; published December 7, 2007.

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Supplemental Material

The following supplementary material is available for this article online:

Figure S1. Mass chromatograms of compounds 1(208), 2 (387) and 3 (647) in the root exudates of wild type and ABC transporter mutants for 3 days' continuous root secretion of compounds (details in Materials & Methods). Arrows indicate the peaks that are absent in the respective mutants. Asterisks indicate the peaks present in wild type. The numbers indicate the peak number. The results represent experiments repeated two times with three replicates each.

Figure S2. Mass chromatograms of compounds 1(208), 2 (387) and 3 (647) in the root exudates of wild type and ABC transporter mutants for 7 days' continuous root secretion of compounds (details in experimental procedures). Arrows indicate the peaks absent in respective mutants. Asterisks indicate the peaks present in wild type. The numbers indicate the peak number. The results represent experiments repeated two times with three replicates each.

Figure S3. Root exudates profile of 21-day-old wild type (Col-0) and ABC transporter mutant *Atpdr4* (PDR4) plants analyzed by HPLC-MS at wavelength 280 nm.

Figure S4. Metabolic profile of Arabidopsis root tissues of 21-day-old wild-type and ABC transporter mutant plants analyzed by HPLC-MS at wavelength 280 nm. Arrows indicate the peaks present or absent in respective mutants. Asterisks indicate the peaks present or absent in the wild type. The numbers indicate the peak number.

Figure S5. Profile of Arabidopsis root tissues of 25-day-old wild-type and ABC transporter mutant plants and analyzed by HPLC-MS at wavelength 280 nm. Arrows indicate the peaks present or absent in respective mutants. Asterisks indicate the peaks present or absent in the wild type. The numbers indicate the peak number.

Figure S6. Chromatogram shows the profiles of root exudates and root tissues from wild type plants. A, root exudates; B, root tissues. The results represent an experiment that was repeated two times with three replicates each.

Figure S7. A, Absorption spectrum of compound 1 showing molecular mass 208 detected by UV coupled with photodiode array detector (DAD). B. Accurate mass and fragmentation pattern of compound 1 detected using a hybrid qudrupole time-of-flight tandem mass spectrometry (QTOF-MS/MS).

Figure S8. Graphs illustrating the representative compounds that show different levels in the ABC transporter mutants compared to wild-type analyzed by GC-MS. * indicates the values are statistically significant ($P<0.01$) compared with wild-type (Col-0) line.

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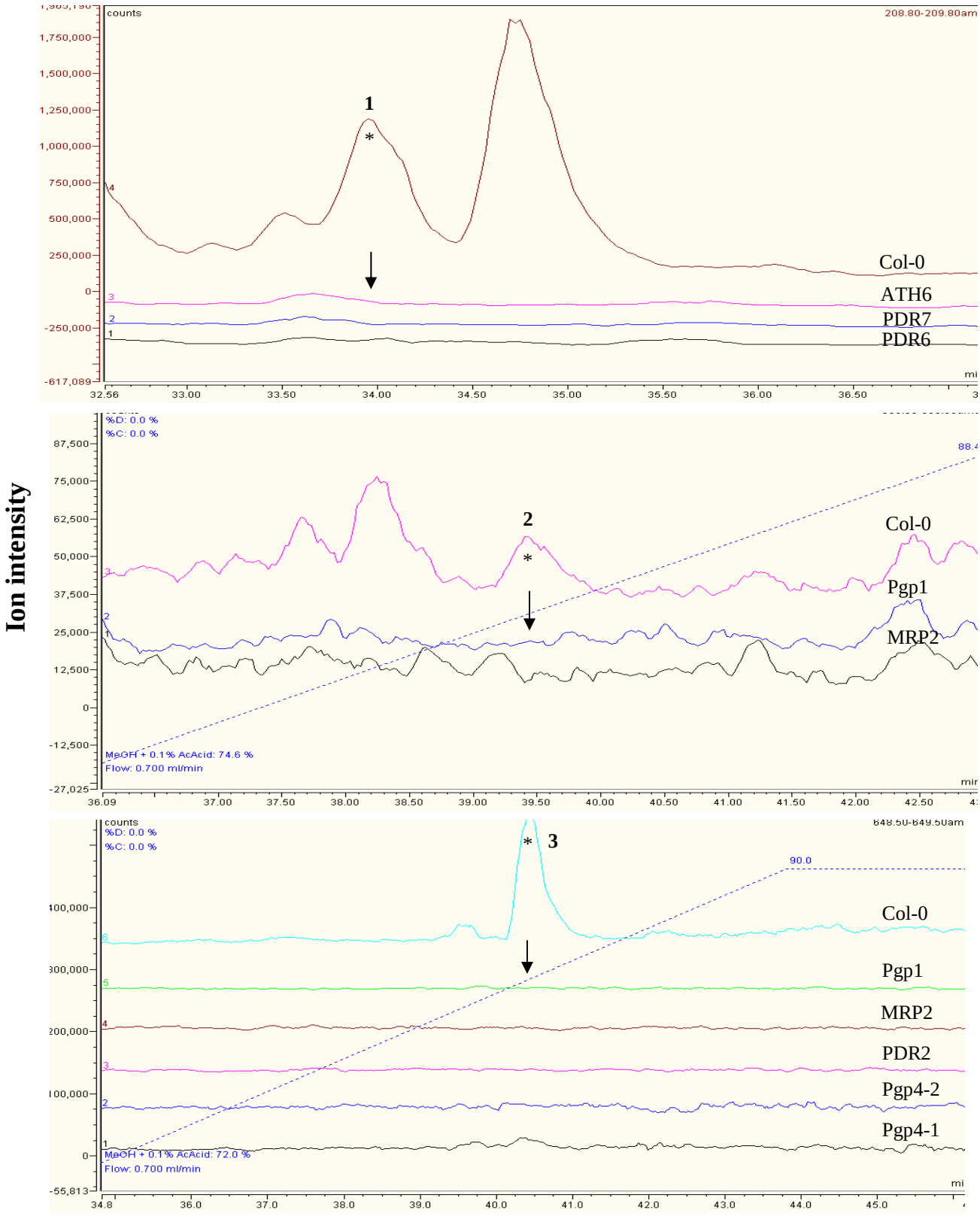
Table S5. List of primers used in this study for RT-PCR assays.

Figure S1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*
Col-0
ATH6
PDR7
PDR6
%D: 0.0 %
%C: 0.0 %
2
*
Col-0
Pgp1
MRP2
MeOH + 0.1% AcAcid: 74.6 %
Flow: 0.700 ml/min
counts
%D: 0.0 %
%C: 0.0 %
* 3
90.0
Col-0
Pgp1
MRP2
PDR2
Pgp4-2
Pgp4-1
MeOH + 0.1% AcAcid: 72.0 %
Flow: 0.700 ml/min
Ion intensity
Time (min)

Figure S2

Ion intensity

Time (min)

Figure S3

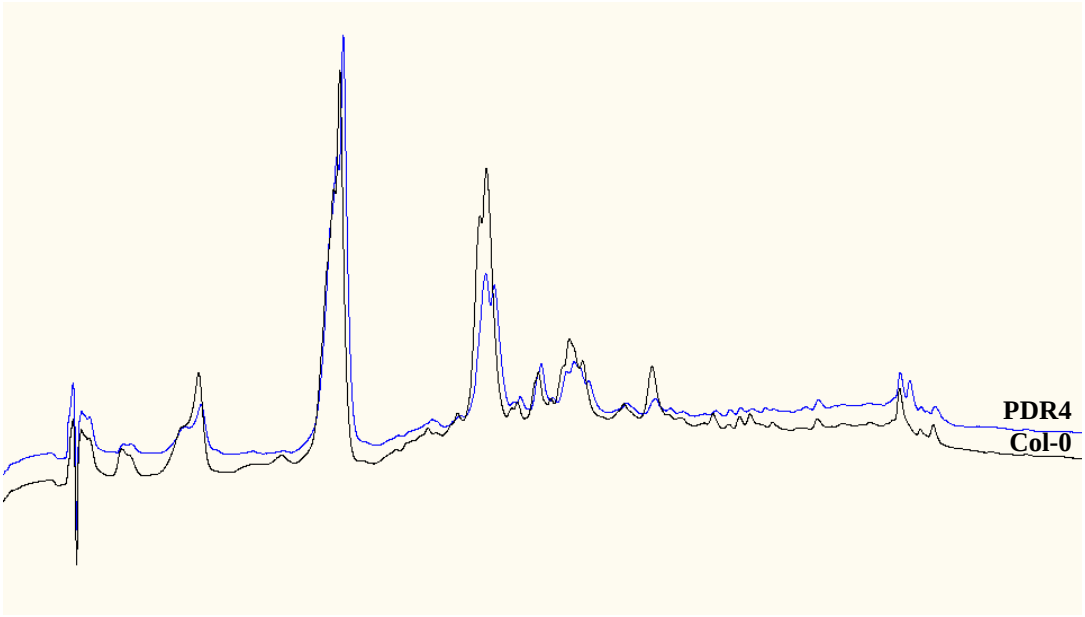
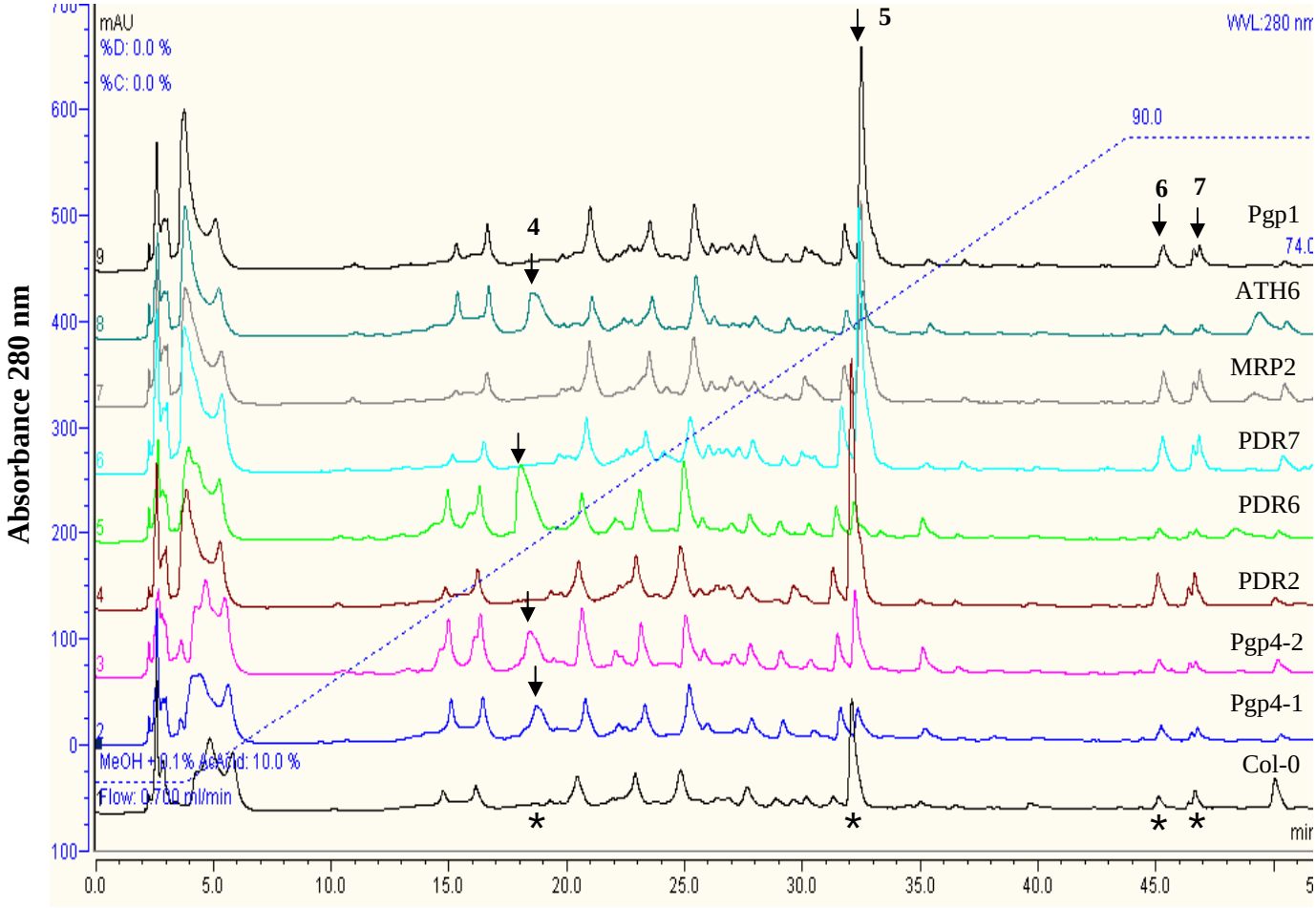


Figure S4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WVL:280 nm
90.0
5
4
6
7
Pgp1
ATH6
MRP2
PDR7
PDR6
PDR2
Pgp4-2
Pgp4-1
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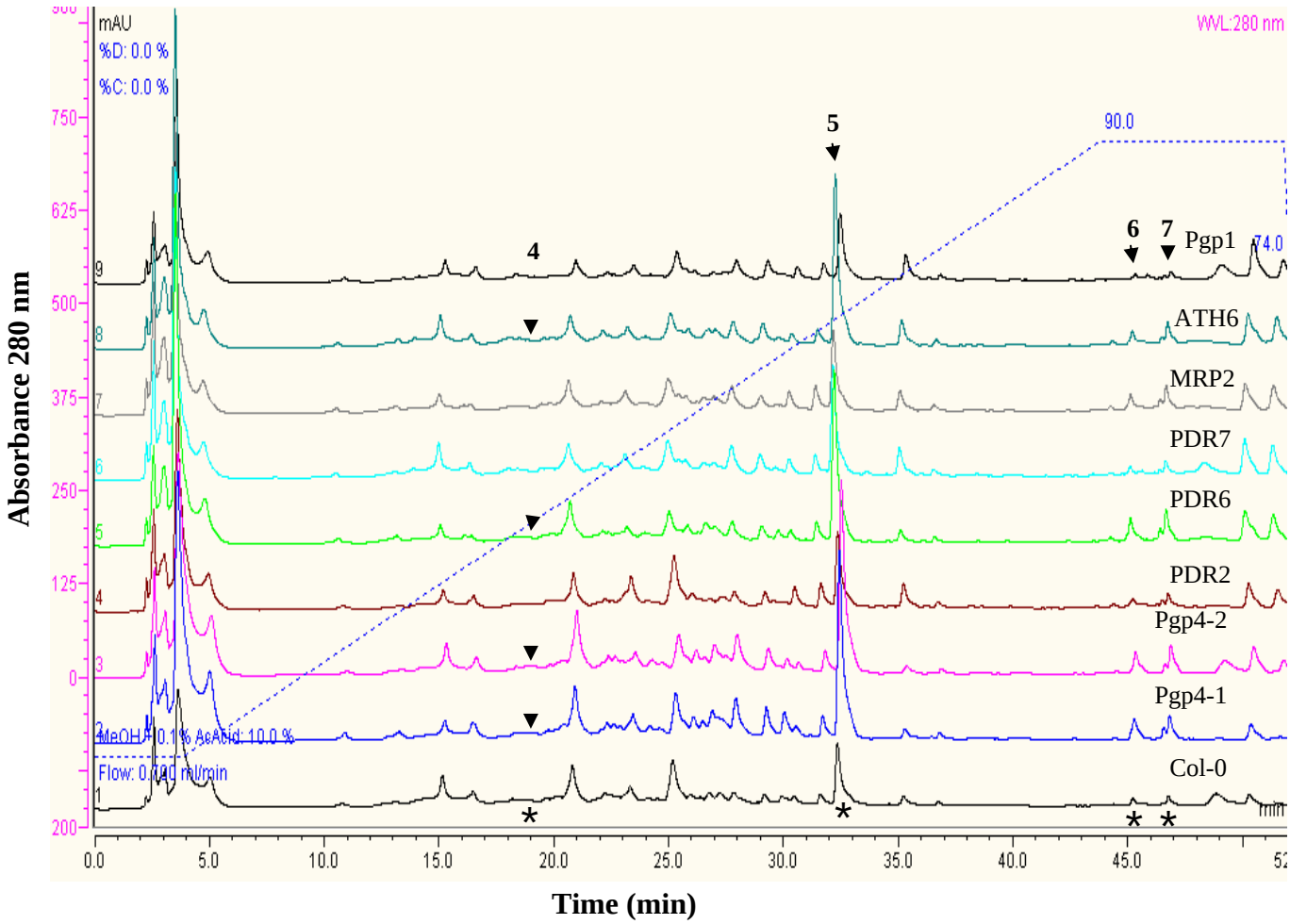
min
Absorbance 280 nm
Time (min)

Figure S5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6
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MRP2
PDR7
PDR6
PDR2
Pgp4-2
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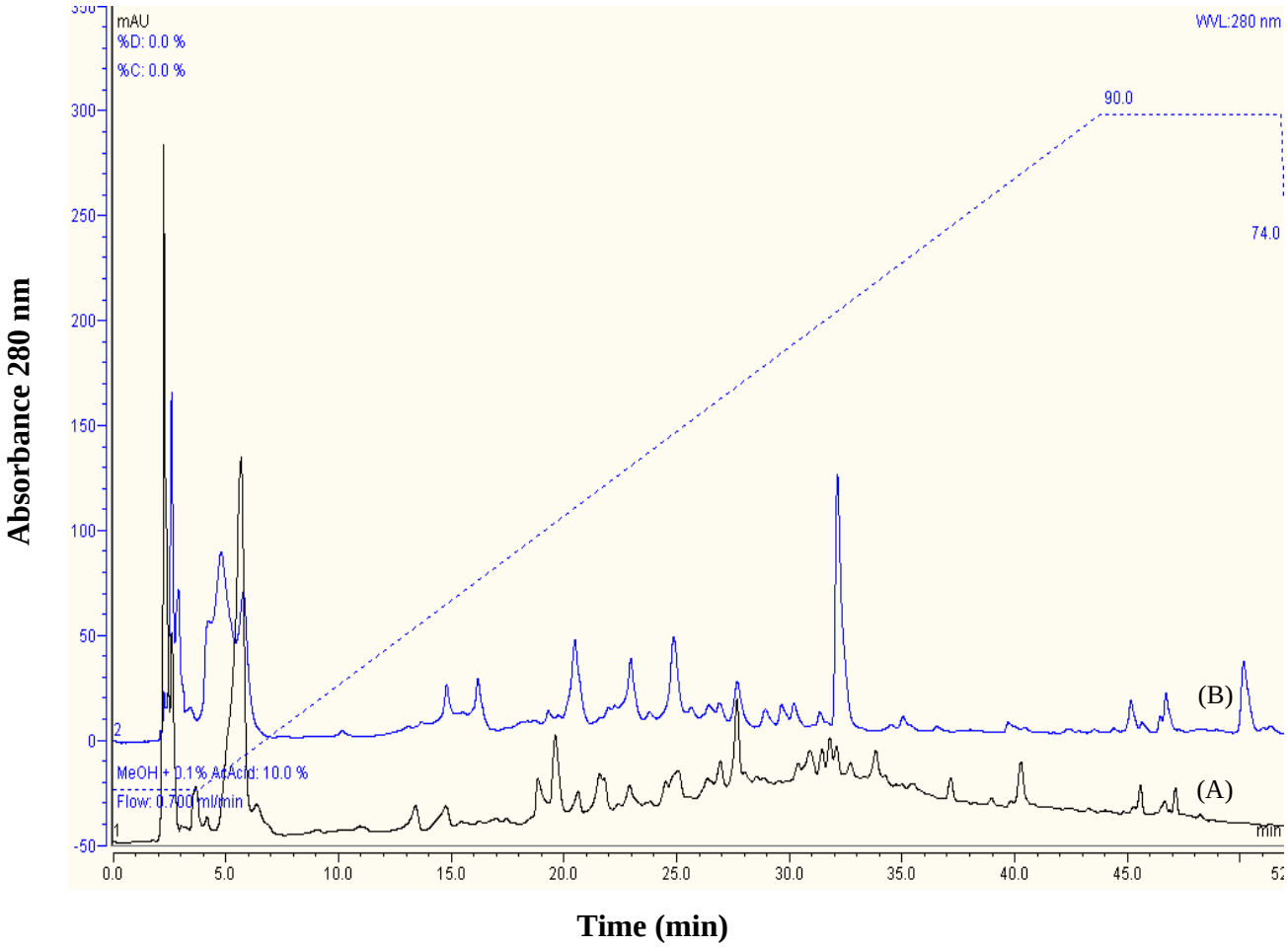
Flow: 0.700 ml/min
Absorbance 280 nm
Time (min)

Figure S6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WVL:280 nm
90.0
74.0
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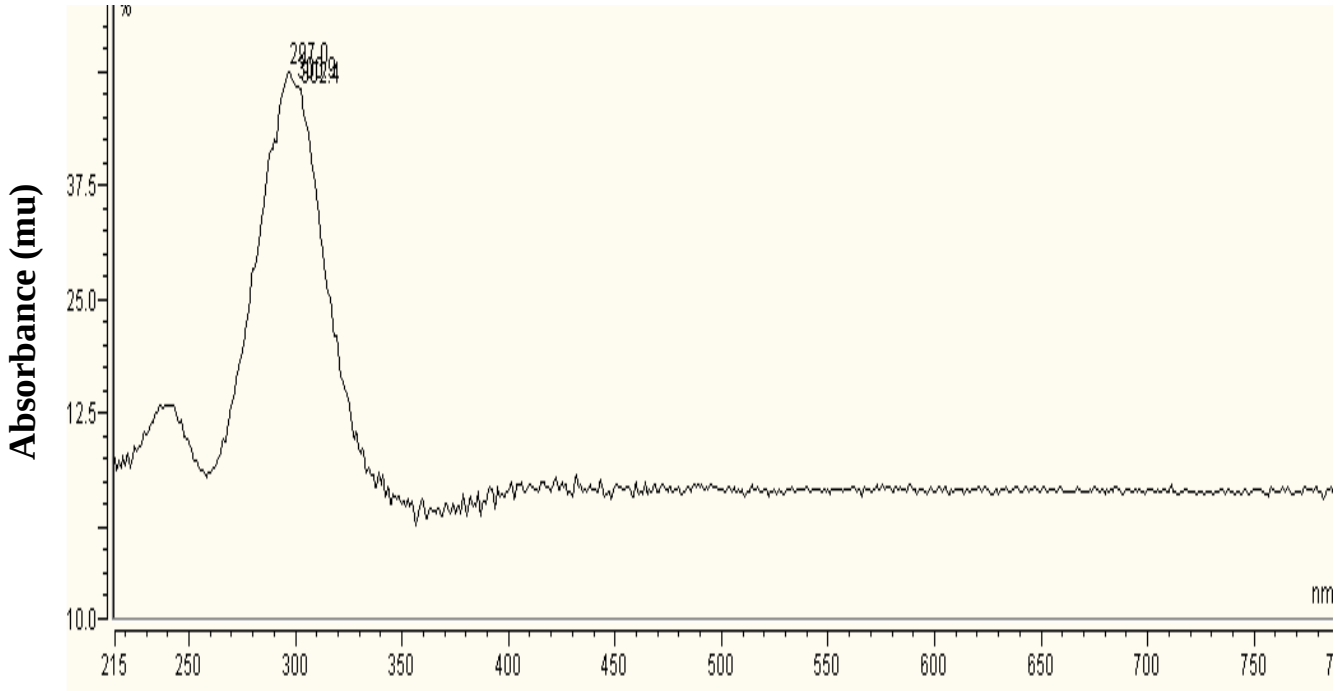
Flow: 0.700 ml/min
min
Absorbance 280 nm
Time (min)

Figure S7

A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B

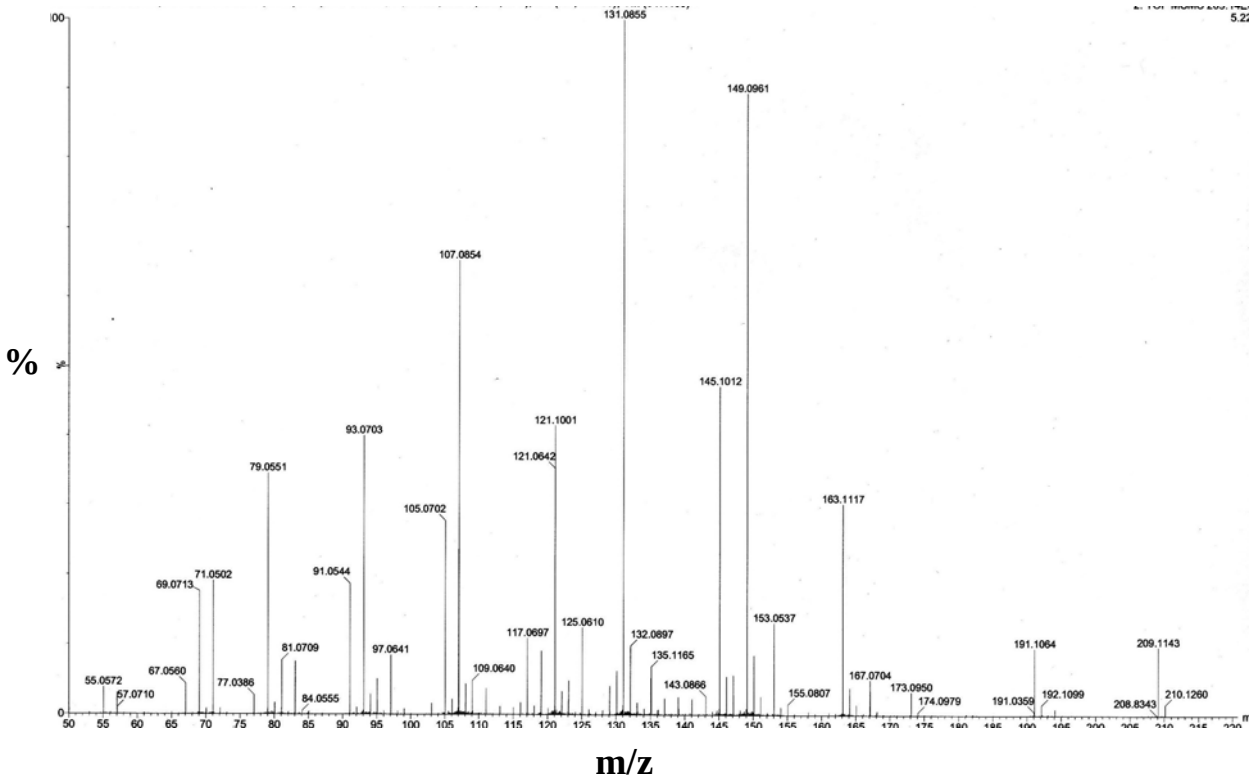
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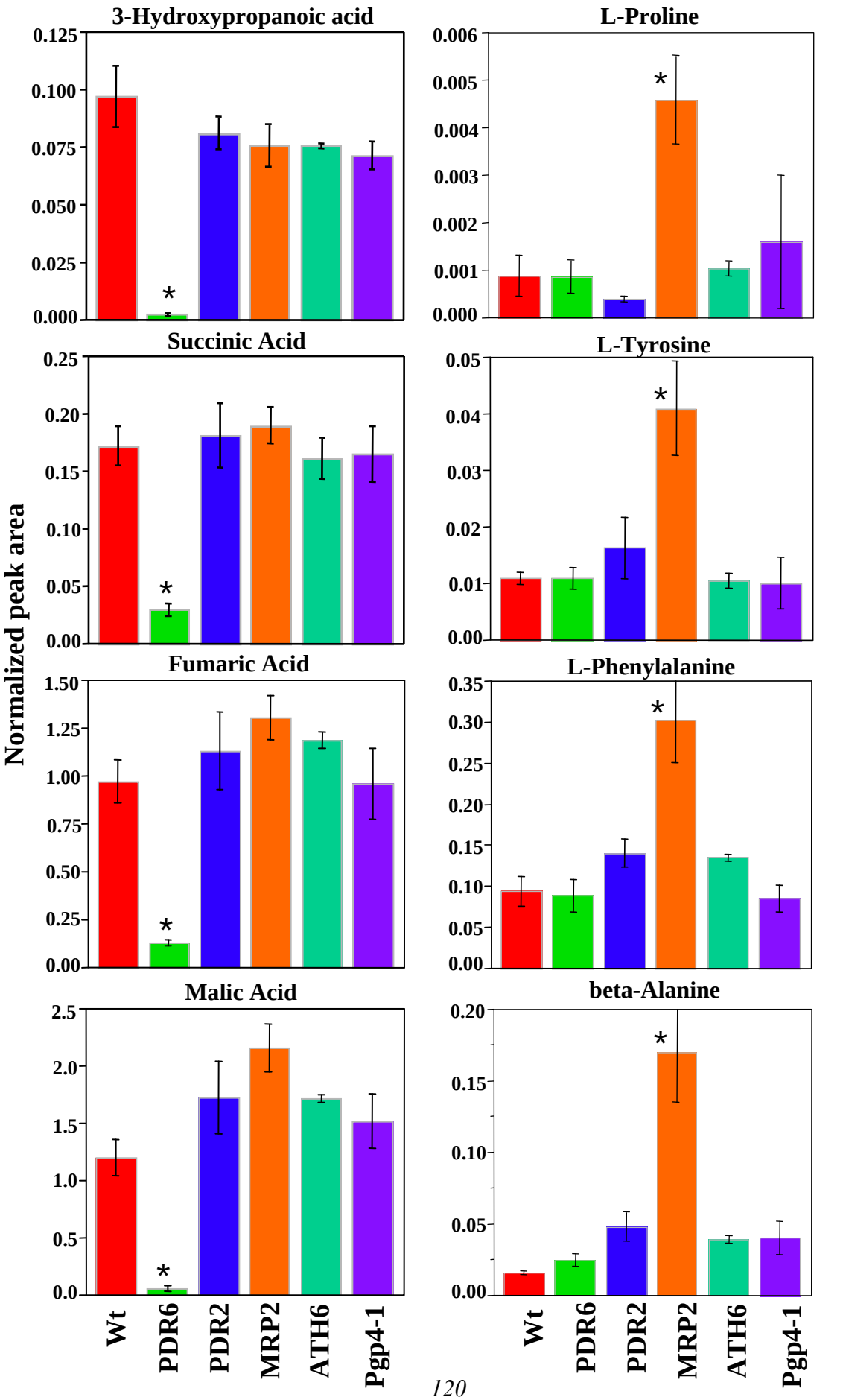
Figure S8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Succinic Acid
L-Tyrosine
Fumaric Acid
L-Phenylalanine
Malic Acid
beta-Alanine
Normalized peak area
Wt
PDR6
PDR2
MRP2
ATH6
Pgp4-1

Table S1
Tissue-and organ-specific expression of 25 ABC transporter genes expressed in Arabidopsis roots

Organ	n	TAP2	PDR2	PDR6	PDR7	PDR8	PDR9	PDR11	MRP2	MRP5	MRP8	WBC1	WBC3	NAP1	NAP3	NAP5	NAP7	NAP9	ATH1	ATH6	ATH10	ATM3	GCN1	GCN3	Pgp1	Pgp4
Genes																										
1 cell suspension	42	1483	49	6200	1220	3949	122	6117	3858	1846	2561	3590	2406	2976	738	1896	1948	1025	1623	681	1793	1830	8697	3719	6306	42
2 seedling	385	504	51	4122	1913	11883	513	1505	1378	1148	991	1202	914	4443	1106	940	2679	2760	1077	460	1018	1383	5523	1939	3018	1707
21 cotyledons	3	765	11	1132	152	15419	221	37	921	850	548	153	766	9597	1772	1032	3530	981	510	304	862	1790	2945	1303	1651	125
22 hypocptyl	3	498	17	1942	2895	10252	273	246	1605	1655	1497	1605	1206	2225	728	674	1516	1283	524	212	1191	1383	4284	1928	3903	653
23 radicle	3	332	16	5732	9220	6162	168	3032	1267	2127	495	2663	1448	1913	1282	459	1138	946	1186	346	1345	1230	5191	2742	3600	2579
3 inflorescence	139	868	28	1902	479	2824	331	362	1550	2867	1341	2261	1292	4778	776	825	1892	1505	1049	312	1473	1785	6467	2143	4155	270
31 flower	58	964	11	1441	177	2770	539	85	1899	2077	1891	4244	1253	4875	1031	616	1745	2041	1392	562	1964	1699	5832	1776	4723	173
311 carpel	6	827	8	1296	207	771	139	31	1165	2158	1467	3410	1725	3427	596	613	1765	881	352	79	1154	1697	6604	2197	5974	59
312 petal	6	1024	9	2213	108	1491	1426	63	1569	2241	2623	7621	1136	4749	701	477	1874	3760	832	118	1785	1890	4289	1687	4601	46
313 sepal	6	2267	10	2013	139	8396	1106	33	4472	2244	3734	5593	613	9299	3113	1367	2761	2099	916	1112	2089	1900	6773	1364	3231	883
314 stamen	8	571	13	573	164	607	274	158	2582	1679	1253	4597	522	3434	1140	461	910	4699	6483	2314	5124	1623	5887	1044	2027	41
3141 pollen	2	303	27	284	132	634	313	103	257	557	468	288	789	1885	231	413	152	6968	20462	7761	6426	1453	3761	329	53	27
315 pedicel	3	842	10	1061	85	5327	202	22	1173	1604	1564	258	1137	7906	328	424	3289	1159	313	151	973	1750	5350	1742	8765	139
32 silique	11	875	7	2114	161	1431	132	117	1645	2618	1027	1353	752	6158	955	393	1702	1192	426	117	1176	1613	3853	1803	3934	119
33 seed	32	873	82	3040	1282	901	162	1290	1090	5563	397	445	1007	4989	317	1670	2187	1265	1134	203	1221	2326	9424	2892	1481	563
34 stem	7	521	16	2114	257	11159	333	40	801	1319	1713	212	775	4221	346	416	1684	873	950	83	819	1175	3512	1535	9173	386
35 node	3	500	8	952	103	4226	142	25	796	3088	2071	185	884	4518	353	582	1910	1266	548	89	849	1481	3971	1583	10654	475
36 shoot apex	17	747	15	1715	549	1566	104	83	1418	2027	928	1155	2638	2559	634	589	1828	756	521	40	1071	1655	7196	2777	4516	83
37 cauline leaf	3	875	9	1660	173	19991	459	70	2932	1239	2524	623	1353	11556	2800	1056	2851	629	997	273	848	1949	4843	1393	2588	540
4 rosette	604	696	19	1971	225	14484	1407	125	1276	962	947	487	985	7250	884	841	2791	1101	655	320	1023	1707	5306	1638	2574	611
41 juvenille leaf	86	842	23	1676	214	9940	1964	254	1222	1052	834	758	1259	5416	865	1232	3135	1810	546	427	1455	1693	8027	2050	2510	1278
42 adult leaf	189	631	25	3328	202	20044	2383	144	930	845	608	689	813	6834	745	690	2591	763	716	408	994	1507	5674	1361	2033	500
43 petiole	12	622	18	721	225	17794	338	24	494	702	748	146	937	5270	599	329	2425	606	405	179	913	1423	3623	1658	3512	143
44 senescent leaf	3	931	11	2027	170	18414	1450	35	4679	1526	2622	226	1505	10440	3701	1365	1752	1076	1026	1485	1013	1404	7782	2218	1609	1138
45 hypocotyl	9	468	17	1892	1751	12185	272	148	1992	3590	6092	83	1663	4332	2658	2310	1132	667	1752	190	1037	1574	11580	1962	3499	1961
451 xylem	3	448	16	331	419	12235	294	132	1847	3624	1331	79	2004	5216	2575	3285	996	674	2602	278	1131	1716	9593	1699	2667	3225
452 cork	3	453	16	1964	2934	15219	279	184	2453	3402	10003	64	1636	4642	2879	2060	1091	573	1604	123	931	1454	13045	1818	4544	1544
5 roots	199	944	66	8748	5766	9226	417	6833	29907	2473	1066	4006	1277	2425	1839	1141	1152	6478	2688	1727	1803	1331	5120	2650	4358	4281
52 lateral roots	4	401	23	7584	7023	12160	306	3062	1837	1872	1311	841	1182	2305	1827	511	1291	9906	1735	1031	1927	1013	3281	1593	4685	2841
53 root tip	4	1808	20	6627	4882	1928	251	8955	2595	1474	1273	2341	3022	2543	742	921	1111	3430	2412	1806	4038	2421	6189	6830	2913	1791
54 elongation zone	7	958	36	8213	9271	4559	309	7058	3079	2755	1906	829	1326	2495	1943	563	1105	13527	1657	1323	4145	1611	4051	3486	4845	4119
55 root hair zone	4	913	42	15160	15413	5787	281	10740	5808	2840	2130	616	846	3000	951	657	794	14391	4300	2345	3925	1681	2796	2335	5197	8427

Represented are the signal intensity values as given by Gene Atlas tool of Genevestigator. “n” denotes the number of chip experiments available by June 2006

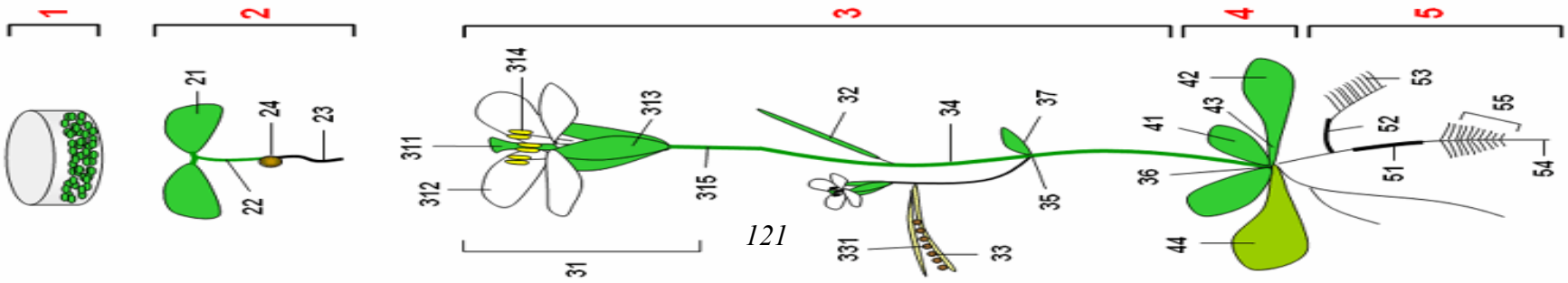

Table S2

Growth stage-dependent expression patterns of 25 ABC transporter genes expressed in Arabidopsis roots

Genes	Plant age (days after germination)								
	1-5	6-13	14-17	18-20	21-24	25-28	29-35	36-44	45-50
TAP2	717	533	864	614	693	659	717	753	834
PDR2	116	51	40	18	17	25	16	10	12
PDR6	3042	4327	4719	2567	1628	3413	1476	1541	2357
PDR7	2491	2223	2565	1010	348	223	400	143	179
PDR8	4154	11433	9838	13338	7033	18150	13007	6894	814
PDR9	164	491	826	309	374	2447	765	383	233
PDR11	2636	1714	3293	880	59	168	134	61	465
MRP2	900	1492	2265	1050	1312	1062	1236	1748	1288
MRP5	1398	1234	1736	961	1346	1054	1087	1918	6310
MRP8	511	1021	1102	815	818	792	1148	1754	609
WBC1	614	1196	2352	471	795	955	994	1120	1859
WBC3	1365	946	1098	1079	1915	857	1165	976	683
NAP1	3880	4418	5214	5162	4845	6675	6760	6053	5670
NAP3	799	1113	1260	993	687	746	1144	1185	469
NAP5	970	964	1268	454	477	732	574	623	1403
NAP7	2663	2650	22062	27748	2547	2446	2660	1877	1719
NAP9	1838	3217	3554	1444	925	1055	1098	1156	1394
ATH1	1180	1126	1629	861	492	729	633	662	2361
ATH6	239	506	976	416	109	435	256	290	701
ATH10	1333	1142	1304	975	1081	1199	1054	1074	1491
ATM3	1847	1420	1639	1455	1820	1555	1607	1523	2070
GCN1	8778	5531	4922	4793	5856	6181	5326	4994	6429
GCN3	3427	2040	2197	1707	2244	1421	1653	1697	1935
Pgp1	2955	3055	3610	2555	3644	2287	3141	5369	2048
Pgp4	1239	1844	2430	822	119	535	487	352	115
n	22	382	360	83	27	188	244	27	28

Depicted are the signal intensity values as given by Gene Chronologer tool of Genevestigator.
"n" denotes the number of chip experiments by June 2006

Table S3

List of ABC transporters and their T-DNA KO mutants used in this study

S.No.	Locus ID	Name	Mutant name	Reference
1	At4g15230	*AtPDR2*	garlic_811_F08*	In this study
2	At2g36380	*AtPDR6*	garlic_627_F11*	In this study
3	At1g15210	*AtPDR7*	Salk_134725	In this study
4	At2g34660	*AtMRP2*	Salk_128332	In this study
5	At3g47780	*AtATH6*	Salk_104795	In this study
6	At2g36910	*AtPgp1*	Salk_046440	In this study
7	At2g47000	*AtPgp4*	Salk_010207 (Pgp4-1)	Santelia et al., (2005)
8	At2g47000	*AtPgp4*	Salk_067557 (Pgp4-2)	Santelia et al., (2005)
9	At2g47000	*AtPgp4*	AtPgp4OX-Pgp4-1	Geisler et al., (2005)

*Currently named as SAIL lines

Table S4

List of primers used in this study for screening homozygous lines of ABC transporters T-DNA KO mutants

Locus ID	Gene	Mutant name	Primer pairs[a]
At1g15210	*AtPDR7*	Salk_134725	LP: 5'-GGCGGTGTCCTTGTTCTACTA-3' RP: 5'-CATCAAAGTTGTCTTACCCGC-3'
At2g34660	*AtMRP2*	Salk_128332	LP: 5'-ACACTAGATGTCCCCTAATGCCAC-3' RP: 5'-GCCAATCTTAGACCTAACAAACAG-3'
At3g47780	*AtATH6*	Salk_104795	LP: 5'-CATGCTACTGGGGTTTAAAGGAAC-3' RP: 5'-CTCGATCAGCAATGATATTGGAAG-3'
At2g36910	*AtPGP1*	Salk_046440	LP: 5'-GTTCGAGGTTTGCAGTGTAGAGAC-3' RP: 5'-TAATGACTCGGAATTCGTCGTATG-'3

[a] LP indicates left gene specific primer and RP indicates right gene specific primer

Table S5

Primers used in this study for RT-PCR assays

Locus ID	Gene	Primer pairs[a]
At4g15230	*AtPDR2*	F: 5'-TGGCAAGAGATGAAGTGTCAGGGAAAG-3'
		R: 5'-CTACAGCAGGATCTGGAATGATTTCTTGG-3'
At2g36380	*AtPDR6*	F: 5'-AGATGTTGACGTCACGAATCTTGCT-3'
		R: 5'-GTTGCCCTGCGTGAAAAGAATTG-3'
At1g15210	*AtPDR7*	F: 5'-GGACATACACGCTTCCCACT-3'
		R: 5'-AAGCACACTTGTTCCCAACC-3'
At2g34660	*AtMRP2*	F: 5'-CCGCAGAAATCCTCTTGGTCTTGATG-3'
		R: 5'-CCTTGTAAGTGGTGTGAGTCATCTTTGG-3'
At3g47780	*AtATH6*	F: 5'-GGGAAACTTGAGAGGGGAAG-3'
		R: 5'-CTTCAGCTCTTTTGGGTTGC-3'
At2g36910	*AtPGP1*	F: 5'-AGACCTGGAAGCGGCACATGC-3'
		R: 5'-TAGAGTCGCGGCTTGTATGAT-3'
At2g47000	*AtPGP4*	F: 5'-TTCATCAGTGGTCTGCAACAG-3'
		R: 5'-TGAAGCTGAACTAACGAAGCA-3'

[a] F indicates forward and R indicates reverse

3.5 MDR-like ABC transporter AtPGP4 is involved in auxin-mediated lateral root and root hair development

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Reprinted from: FEBS letters (2005) **579**: 5399-5406

In this work, we characterized the phenotype of two *AtPGP4* T-DNA insertion mutants. We provided several lines of evidences suggesting that AtPGP4 is involved in root development and in auxin transport processes as previously shown for AtPGP1. In contrast to PGP1, the reduced root IAA uptake capacities of the two mutants compared to the wild type, as well as the fact that AtPGP4 confers hypersensitivity to the IAA sensitive *yap1-1* yeast mutant, suggests that PGP4 acts as an uptake protein.

Our data demonstrated for the first time that an ABC transporter catalyzes the cellular import of auxin. A month after our work was published, new data about AtPGP4 became available supporting our findings (Terasaka et al., 2005).

MDR-like ABC transporter AtPGP4 is involved in auxin-mediated lateral root and root hair development

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Received 19 July 2005; revised 24 August 2005; accepted 25 August 2005

Available online 16 September 2005

Edited by Julian Schroeder

Abstract Previous data have suggested an involvement of MDR/PGP-like ABC transporters in transport of the plant hormone auxin and, recently, AtPGP1 has been demonstrated to catalyze the primary active export of auxin.

Here we show that related isoform *AtPGP4* is expressed predominantly during early root development. *AtPGP4* loss-of-function plants reveal enhanced lateral root initiation and root hair lengths both known to be under the control of auxin. Further, *atpgp4* plants show altered sensitivities toward auxin and the auxin transport inhibitor, NPA. Finally, mutant roots reveal elevated free auxin levels and reduced auxin transport capacities. These results together with yeast growth assays suggest a direct involvement of AtPGP4 in auxin transport processes controlling lateral root and root hair development.

Keywords: Auxin transport; ABC transporter; MDR; *p*-Glycoprotein; Lateral root; Root hairs

1. Introduction

Despite its controversially discussed action as hormone, morphogen or neurotransmitter-like substance [1] auxin has proven as unique signaling molecule virtually controlling all plant developmental processes [2–4]. Recent research has concentrated on the polar transport of auxin, principally indole-3-acetic acid (IAA). Polar auxin transport (PAT) is regulated at the cellular level and is apparently both a product and determinant of cellular polarity [1,2,5]. Auxin loading and unloading is thought to be mediated by protein complexes that are characterized by members of the AUX and PIN protein families; the latter have been suggested to function as gradient-driven auxin influx and efflux carriers, respectively, but a clear biochemical verification is still lacking [5,20].

Previous findings have provided intriguing evidence that ABC transporters of the multidrug resistance (MDR)-like *p*-glycoprotein (PGP) subclass [6,7] are involved in auxin transport [8–11]. First, several MDR/PGPs (hereafter referred to as PGPs) found in *Arabidopsis* specifically bind the auxin efflux inhibitor 1-N-naphtylphtalamic acid (NPA, [12]). Second, the phenotype of *atpgp19* (*atmdr1*) and *atpgp1 atpgp19* T-DNA insertional null mutants and *atpgp1* antisense plants show a pleiotropic auxin-related phenotype [10,30]. Additionally, basipetal auxin transport in the hypocotyl of *atpgp1* and *atpgp19* single and double mutants is reduced [8,11]. Finally, loss of an *AtPGP1* ortholog in maize *br2* and sorghum *dw3* mutants leads to compact stalks and reduced basipetal IAA transport [13].

PIN1 has been shown to be mislocalized in Arabidopsis *pgp* mutants [9]. Therefore, in mechanistic models, it has been proposed that PGPs function in PIN vesicular trafficking [9,10], PIN regulation, or direct transport of auxin [9]. Very recently, AtPGP1 has been demonstrated by heterologous expression in yeast and mammalian cells to catalyze the primary active efflux of natural and synthetic auxin and oxidative breakdown products [11]. AtPGP1-mediated efflux is sensitive to auxin efflux and ABC transporter inhibitors. Moreover, AtPGP1 exhibits non-polar expression in the root and shoot apices and polar (mainly basal [36]) localization in mature cortical and endodermal cells in the distal elongation zone suggesting a function in polar or reflux movement of auxin [11,14]. In agreement, basipetal auxin transport from the root tip is strongly reduced. As a result auxin levels are reduced in *atpgp1* mutant plants as shown by gas chromatography–coupled mass spectrometry (GC–MS) analysis and reduced expression of the auxin responsive *DR5::GUS* reporter gene [11,15].

Genetic and physiological evidence suggests that auxin is required at several specific developmental stages to facilitate root hair (RH) development and lateral root (LR) formation [16,22,23,25]. While an interpretation for root hair development is complicated by interaction among hormone response pathways [21], a shoot-derived auxin pulse has been suggested to promote the emergence of LR primordia [17]. Lately, in a microarray-based approach to profile early lateral root initiation, a homologue of *AtPGP1*, *AtPGP4*, was shown to be induced 2 h after external lateral root induction [18]. Here we

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Abbreviations: PAT, polar auxin transport; IAA, indole-3-acetic acid; NPA, 1-*N*-naphtylphtalamic acid; LR, lateral root; RH, root hair; ACC, aminocyclopropane carboxylic acid; AVG, ethylene biosynthesis inhibitor aminovinylglycine; GC–MS, gas chromatography-coupled mass spectrometry; MS, Murashige and Skoog basal medium; SE, standard error; ORF, open reading frame

0014-5793/$30.00
doi:10.1016/j.febslet.2005.08.061

show that loss-of-function mutant alleles of *AtPGP4* reveal more lateral roots and longer root hairs at early stages. Moreover, mutant plants show elevated auxin levels but reduced auxin uptake capacities suggesting that AtPGP4 is involved in auxin transport processes that trigger plant lateral root and root hair initiation.

2. Materials and methods

2.1. Isolation of atpgp4 mutants

In order to identify mutant lines carrying T-DNA insertions in the *AtPGP4* gene (At2g47000), two T-DNA insertion lines SALK_010207 and SALK_067557 were ordered from the Arabidopsis Stock Center at Ohio State University (ABRC). Segregation of T-DNA insertion was shown by PCR employing the following combinations of gene- and T-DNA-specific primers: gene-specific primers *AtPGP4* (for SALK_010207 fw: 5′-tagtttacctttgcatcagcg; rev: 5′-aaaggacccggaaggagctta; for SALK_067557 fw: 5′-ccaagcgacaaattaatgtcgag; rev: 5′-acgcgctagaccgtgttatgg). PCR reactions were performed on genomic DNA using these primers in combination with LBb1 5′-GCGTGGACCGCTTGCTGCAACT which anneals to the left border of the T-DNA insertion, according to the protocol published at http://signal.salk.edu/tabout.html. T-DNA insertion numbers were determined by Southern Blot analysis. Genomic DNA from wt and pgp4 mutants was digested with *Hin*dIII and *Bam*HI. The resulting blot was probed with a [α-^{32}P]dCTP-labeled T-DNA specific DNA fragment corresponding to the left border region of the T-DNA with a size of 266 bp. The probe was obtained by PCR amplification using the following pairs of primers: pROK2fw (5′-AACAGCTGATTGCCCTTCAC) and pROK2rev (5′-TTTGGGTGATGGTTCACGTA).

2.2. Semi-quantitative RT-PCR

For tissue-specific expression analysis, RNA was extracted from leaves, roots, flowers and siliques of pot-grown plants. Seedlings were grown on bactoagar for seven days and each day seedlings were collected. Total RNA was purified from plants using the RNeasy Plant Mini Kit (Qiagen). cDNAs were prepared using M-MLV reverse transcriptase (Promega) as indicated by the manufacturer. Semi-quantitative RT-PCRs were performed as described in [32] using gene-specific sense (S) and anti-sense (AS) primers for *PGP4* (PGP4-S: 5′-ttc atc agt ggt ctg caa cag; PGP4-AS: 5′-tga agc tga act aac gaa gca), ACTIN2 (actin2-S: 5′-tggaatccacgagacaaccta; actin2-AS: 5′-ttctgtgaacgattcctggac), and S16 (S16-S: ggcgactcaaccagctactga and S16-AS: cggtaactcttctggtaacga).

2.3. Plant growth conditions

Seeds were surface sterilized for 5 h in a chamber containing vapor of HCl and Na-hypochlorite and stratified in a 0.1% agar solution for two days at 4 °C. Subsequently, the seeds were plated on sterile half-strength Murashige and Skoog (MS) medium containing 2% sucrose solidified with 0.6% phytagel (Sigma, Buchs, Switzerland), and grown vertically as described in [35]. For phenotypes of adult plants, mutants were grown under short day conditions: 8 h, 100 μmol m^{-2} s^{-1} white light, 22 °C.

2.4. Morphometric analysis

Seedlings were grown vertically as described above on nutrient media optionally supplemented with 10 nM IAA, 5 μM NPA, 1 μM AVG or 200 nM ACC. Five dag seedlings were photographed using a Leica DMR microscope equipped with a Leica DC300 F charge coupled device (CCD) camera and root hair lengths all along the primary root were measured from digital pictures using the tool "Image Manager" of the Leica IM1000 software (Leica, Heerbrug, CH). Lateral roots number of 40 seedlings per each genotype was counted after 5, 7, 9 or 11 dag under a dissecting microscope. Values were used to determine the means (±S.E.). The measurement was repeated two times showing similar results. *P* values were analyzed by Student's *t* test. Root hairs and lateral roots were photographed using a Nikon SMZ1500 binocular, equipped with a Nikon Coolpix 5000 camera. Digital pictures were processed using Adobe Photoshop 7.0 (Adobe Systems).

2.5. Auxin measurements

For free IAA quantification, root and shoot segments of 30–50 seedlings were collected, pooled and their fresh weight was determined. Samples were methanol extracted and analyzed by GC–MS. Calculation of isotopic dilution factors was based on the addition of 100 pmol $^2H_2$-IAA to each sample. Measurements are presented as means of two individual samples.

2.6. Root auxin transport assays

Arabidopsis seedlings were grown in hydroponic culture as described in [35]. Two weeks later seedlings were collected and incubated in 10 ml transport buffer (5 mM HEPES, pH 7.0, and 1 mM $CaCl_2$) supplemented with 5-^{3}H-IAA (specific activity 20 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO, 1 μl/10 ml). At 2, 8, 16, 24, 32, 40, 48 min, seedlings were washed in transport buffer containing 10 μM cold IAA and the amount of radioactivity in the first 1 cm of root was quantified by scintillation counting. Two-minute treatments were considered as initial loading and used to calculate relative transport kinetics. Experiments were repeated at least three times, representative results are shown.

2.7. Recording of root apex auxin fluxes using an IAA-specific microelectrode

A platinum microelectrode as a substrate electrode immobilizing multiwalled carbon nanotubes was used to monitor IAA fluxes as described in [33]. For measurements, plants were grown in hydroponic cultures and used 5 dag.

2.8. Yeast yap1-1 complementation assays

AtPGP4 was cut out from pBSKII-AtPGP4 (kindly provided by A. Murphy) and inserted *Kpn*I/*Not*I into yeast shuttle vector pYES2 (Invitrogen) resulting in pMG89. pMG89 was introduced into *S. cerevisiae* strains JK93da and *yap1-1* [31], respectively, and single colonies were grown in synthetic minimal medium without uracil, supplemented with 2% glucose (SD-URA). For detoxification assays, transformants grown in SD-URA to an OD_{600} = 0.8 were washed and resuspended in water to OD_{600} = 1 in water. Cells were 5-times 10-fold diluted and each 5 μl were spotted on minimal media galactose plates (SG-URA) containing either 250 μM 5-fluoro-indol (5-FI, JK93da) or 10 μM IAA (*yap1-1*). Pictures were taken after 3–5 days of growth at 30 °C. Assays were performed with three independent transformants.

3. Results and discussion

3.1. Expression of AtPGP4

Based on microarray transcript profiling, AtPGP4 has been suggested to be involved in early lateral root initiation [18]. Additionally, Digital Northern analysis (www.genevestigator.ethz.ch, [26]) supported a predominant expression of *AtPGP4* in the root while expression in aerial parts of the plant is significantly lower (Suppl. Fig. 1A). Interestingly, expression is high both in the root elongation zone and in lateral roots. Furthermore, again based on a microarray surveys *AtPGP4* seems to be indeed expressed at early stages of the plant development (Suppl. Fig. 1B). We confirmed these *in silico* data by semi-quantitative RT-PCR. Using *AtPGP4* gene-specific primers we found high expression in roots while expression in other parts of the plants was low (Fig. 1A). Likewise, *AtPGP4*-specific transcripts accumulate at early stages of plant development with a maximum expression at day 3 (Fig. 1A).

3.2. atpgp4 mutants show increased lateral root formation and reduced sensitivity toward auxin transport inhibitor NPA

For phenotypic analysis, two SALK T-DNA lines [19] were obtained and insertions in homozygous plants were verified by PCR (Fig. 1C). *atpgp4-1* (SALK_010207) carries a T-DNA

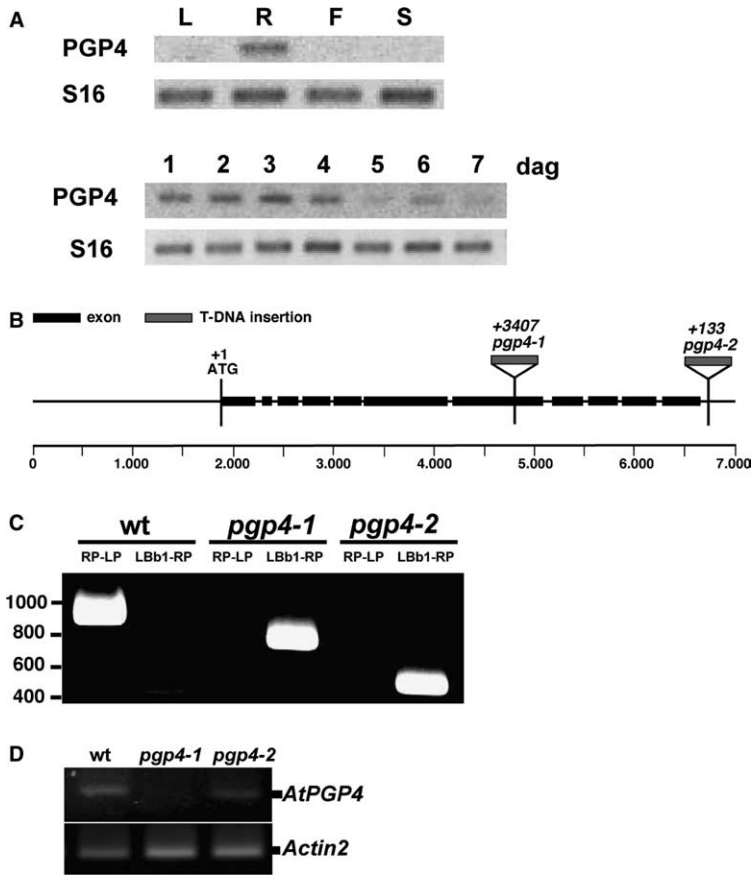

Fig. 1. Expression and mutant analysis of *AtPGP4*. (A) Results of RT-PCR analysis suggest that AtPGP4 is expressed in low amounts in the roots (upper panel) and at early stages of seedling development (lower panel). cDNAs from different tissues or developmental stages were synthesized from total RNA and AtPGP4 and 40S ribosomal subunit S16 (internal control) specific transcripts were detected by PCR. (B) Genomic structure of *AtPGP4* and T-DNA insertion sites. Start of open reading frame (ORF) is indicated by ATG; T-DNA insertions positions of mutant lines SALK_010207 (atpgp4-1) and SALK_067557 (atpgp4-2) are marked as nucleotide distances from +1 (ATG) and from the stop codon, respectively (see Section 3). (C) PCR verification of T-DNA insertions using primer combinations as described in Section 2. (D) RT-PCR verifies the absence in the atpgp4-1, but a low amount of transcript in the atpgp4-2 mutant. cDNAs were synthesized from total RNA extracted from root of 5 dag seedlings, and AtPGP4- and actin2 (internal control) specific transcripts were detected by PCR.

insertion in exon 7 (at position 3407 bp) and *atpgp4-2* (SALK_067557) in the 3′ UTR (133 bp downstream of the stop codon, see Fig. 1B). While for *atpgp4-1* no transcript was detected by semi-quantitative RT-PCR (Fig. 1D), it was markedly reduced in *atpgp4-2*. This is further supported by the phenotypic analysis presented below, which shows no significant differences between the two mutant alleles. Both mutants contained only single T-DNA insertions as was verified by Southern blot analysis (results not shown).

While pot-grown *atpgp4* mutant plants performed no obvious growth phenotype (results not shown), the strong expression in radical parts of the plant prompted us to investigate root growth in more detail. When grown on agar plates both mutant alleles of AtPGP4 showed slightly enhanced primary root growth at five days after germination (dag) compared to the wild-type while significant differences were only found for *atpgp4-1* (Suppl. Fig. 2).

We quantified lateral root initiation, and found that atpgp4 seedlings after 5 dag developed significantly more LR primordia (137%) compared to the wt (Fig. 2A, Table 1). This difference diminishes after 7 and 9 days, which is inline with the expression profile for *AtPGP4*. LR formation is apparently induced by an endogenous and temporary shoot derived auxin burst [16,17,25]. In order to investigate if these differences in LR formation were directly a consequence of altered auxin levels, we tried to manipulate plant endogenous auxin levels by addition of low concentrations of IAA and the auxin transport inhibitor NPA. Exogenous IAA is known to inhibit root elongation but stimulates LR formation [16]. In agreement, growth at low concentration of exogenous IAA (10 nM) enhanced LR formation in wt at 5 dag thus mimicking the developmental defect of the atpgp4 mutants (Table 1). LR induction by IAA at 5 dag is significantly weaker in both atpgp4 mutant alleles (126%) compared to the wt (137%) while at later stages on IAA leads to a reduced induction both in wt and *atpgp4* (Table 1, Fig. 2B) until relative LR numbers wt and atpgp4 are indistinguishable after 9 dag.

The auxin transport inhibitor NPA has become an important pharmacological tool in auxin research as is known to block polar auxin transport, to bind to putative auxin transport complexes [12] and to mimic the pin-shaped mutant phenotype of putative auxin efflux facilitator PIN1 [2,4]. Recently, it was shown that NPA binds to Arabidopsis PGPs [9,12] and that NPA efficiently inhibits AtPGP1-mediated auxin transport [11]. Here we show that NPA reduces LR formation in the wt – verifying previous data [16,25] – but that inhibition

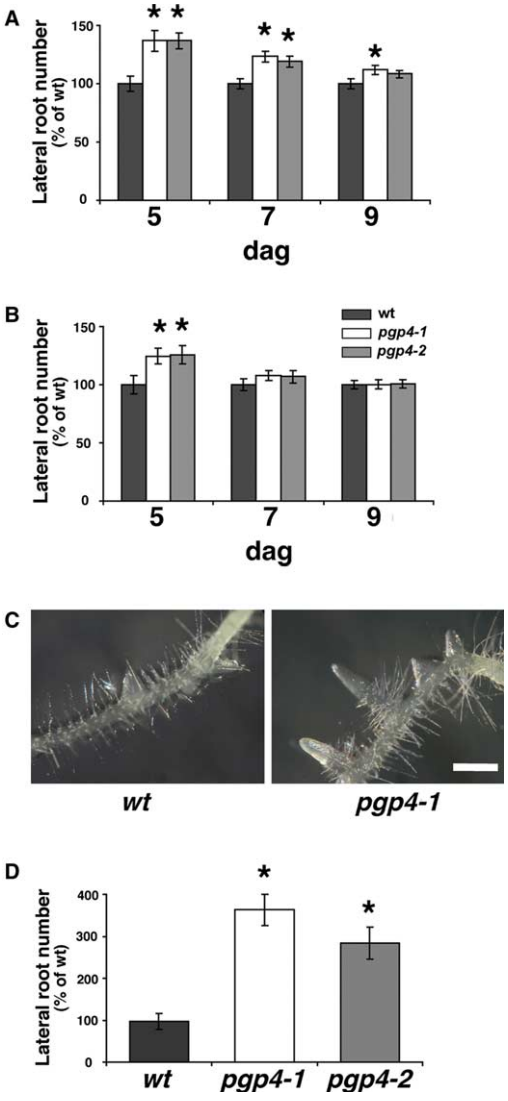

Fig. 2. atpgp4 mutants show enhanced lateral root formation and reduced sensitivity to the auxin transport inhibitor, NPA. (A) Lateral root formation is significantly enhanced in atpgp4 mutant alleles at 5 dag but decreases at later stages. (B) Lateral root induction by IAA at 5 dag is significantly weaker in both atpgp4 mutant alleles compared to the wt, while at later stages, IAA leads to a reduced induction both in wt and atpgp4 (see Table 1 for absolute values). (C) Lateral root phenotype of atpgp4-1 grown in the presence of 5 μM NPA for 11 days. Atpgp4 mutants are less sensitive to the inhibitory effect of NPA. Scale bar = 500 μm. (D) Lateral root formation in atpgp4 mutants is 3–4 times enhanced compared to the wt. Lateral root number of vertically grown plants (40 seedlings each) in the presence or absence 10 nM IAA (determined at dag 5, 7 and 9) or 5 μM NPA (11 dag). Shown are mean numbers ± S.E., significant differences to wt growth (*P* values analyzed by Student's *t* test) are marked with an asterisk. Absolute values are presented in Table 1.

is lower in the atpgp4 mutants (Fig. 2C, Table 1) leading to a drastic (3–4-fold) enhanced relative LR number (Fig. 2D). We also found that LR shape is distorted (Fig. 2C) and that LR emergence site is shifted nearby the root apex (data not shown). Our data indicate that external IAA reduces enhanced LR formation in atpgp4 while NPA increases this difference pointing to an involvement of AtPGP4 in auxin homeostasis of the root.

Table 1
atpgp4 mutants show enhanced lateral root formation and reduced sensitivity to the auxin transport inhibitor, NPA

Treatment	Dag	Wt	pgp4-1	pgp4-2
control	5	3.33 ± 0.22	4.55 ± 0.29	4.55 ± 0.23
	7	12.12 ± 0.54	14.95 ± 0.58	14.42 ± 0.58
	9	30.35 ± 1.29	34.025 ± 1.18	32.9 ± 1.02
IAA	5	4.57 ± 0.34	5.7 ± 0.31	5.75 ± 0.35
	7	12.87 ± 0.69	13.85 ± 0.54	13.75 ± 0.66
	9	29.88 ± 1.03	29.98 ± 1.08	30.00 ± 1.07
NPA	11	1.63 ± 0.32	6.08 ± 0.6	4.75 ± 0.63

Lateral root number of vertically grown plants (40 seedlings each) in the presence or absence of 10 nM IAA or 5 μM NPA. Shown are mean numbers ± S.E. Relative values are presented in Fig. 2.

3.3. Root hairs of atpgp4 mutants are longer and more variable in length

Like for LRs, RH initiation and elongation is well known to be regulated by auxin [22]. The growth defect of LRs in the atpgp4 mutants thus prompted us to investigate RH number and lengths in atpgp4. While both *atpgp4* alleles have similar RH numbers compared to the wt (Fig. 3A), RHs of the mutant were longer (136%, Fig. 3D) and more variable in lengths (Fig. 3B) under control conditions.

It has been shown that growth in the presence of either auxin or auxin transport inhibitors results in enhanced root hair length [21,22]. Here we report that exogenous IAA stimulated RH elongation in wt and atpgp4 seedlings (Fig. 3B). However, like for the LRs, enhanced RH elongation was reduced in the mutant (118%) when compared to the wt (133%). In contrast, growth on NPA leads to enhanced RH length similarly in wt and atpgp4 (Fig. 3D).

Seedlings grown on NPA plates showed constant differences in RH length between wt and atpgp4 on control and NPA plates (between 135% and 141%), while differences on IAA plates were lower (around 118%). Interestingly, mutant RH on NPA displayed a far flatter and non-Gaussian distribution in length comparing to the wt (Fig. 3B).

Genetic and physiological evidences implicate that auxin cross talks with ethylene in promoting root hair elongation [21,22]. In order to dissect the nature of this effect, and in order to test an involvement of AtPGP4 in growth defects caused by an individual plant hormone we repeated root hair measurements in the presence either of ethylene precursor aminocyclopropane carboxylic acid (ACC) which induces ectopic root hair formation or ethylene biosynthesis inhibitor aminovinylglycine (AVG) which abolishes root hairs, respectively [21]. In our experiments we found indeed that 200 nM ACC dramatically enhances RH lengths (Fig. 3C) while 1 μM AVG blocks efficiently RH elongation (Fig. 3A and C). This was true for both wt and atpgp4; however, as found for experiments using exogenous manipulation of auxin levels, both treatments lead to a flatter and more unequal distribution (Fig. 3C). Further, our data indicate that atpgp4 plants are even under low endogenous ethylene able to develop a population of root hairs that are as long as under control conditions (Fig. 3A and C).

Interestingly, both exogenous up- and down-regulation of intracellular ethylene by ACC and AVG, respectively, reverts the mutant phenotype (enhanced RH length). Under control conditions relative root hair length of atpgp4 is enhanced to around 136% while on ACC (wt: 180%, *atpgp4*: 165%) and AVG (wt: 30%, *atpgp4*: 37%) differences are smaller. This is

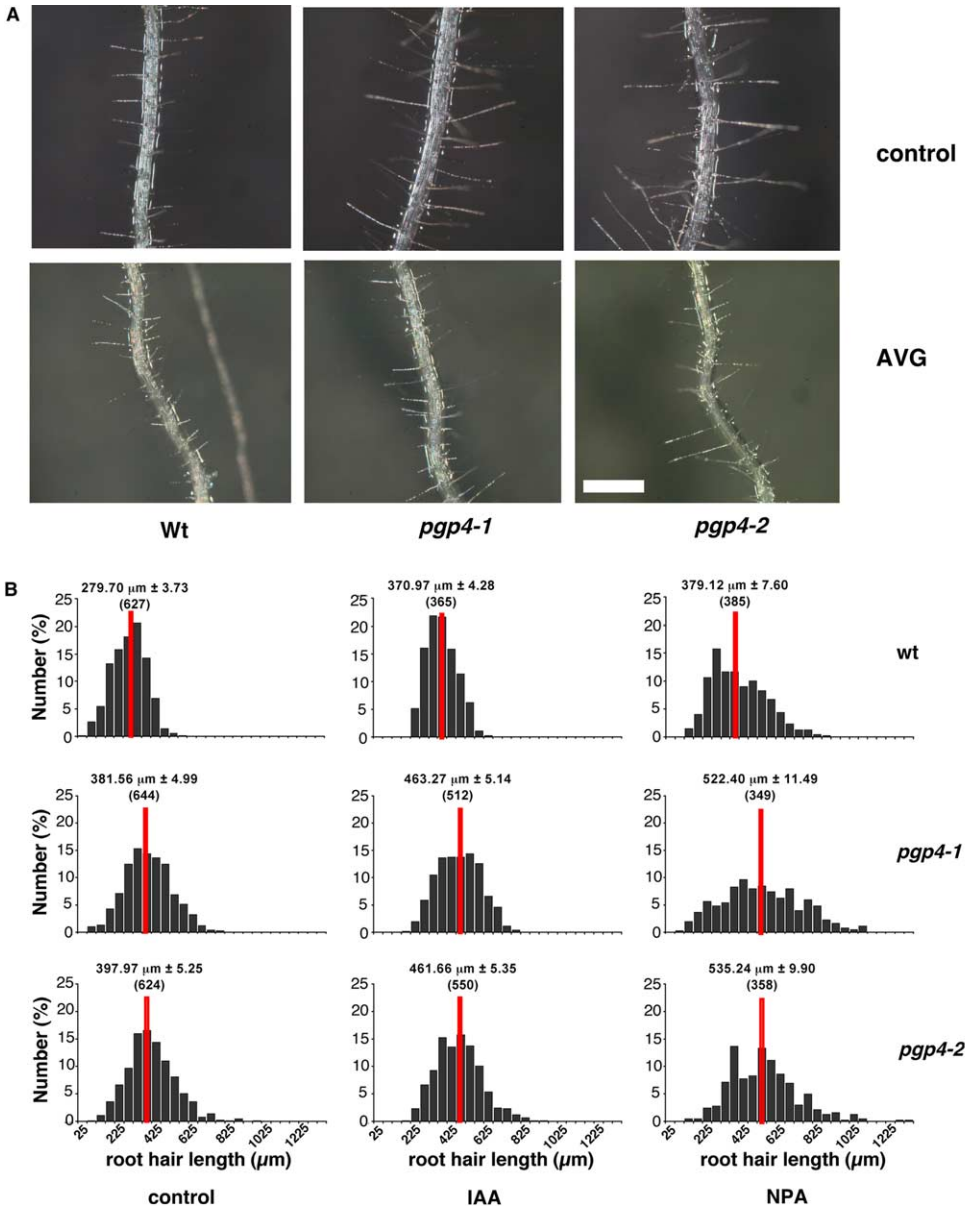

Fig. 3. Root hairs of atpgp4 mutants are longer and more variable in length. (A) Root hair (RH) phenotype of atpgp4 mutants grown on unsupplemented media (upper panel) or on 1 μM AVG (lower panel) for five days. Scale bar = 500 μm. (B) Atpgp4 mutants have longer RHs and their distribution is flatter compared to the wt. On IAA enhanced RH elongation was reduced in the mutant compared to the wt. (C) In wt and atpgp4 seedlings, ACC enhanced RH lengths, while AVG efficiently blocked RH elongation. (D) Comparison of relative root hair lengths between wt and atpgp4 mutants seedlings grown for 5 days in the presence of 10 nM IAA, 5 μM NPA, 1 μM AVG or 200 nM ACC. Distribution of root hair lengths in 5-day-old seedlings grown on unsupplemented media (control), 10 nM IAA, 5 μM NPA, 1 μM AVG or 200 nM ACC. Values are mean numbers ± S.E. (*P* values analyzed by Student's *t* test). In parenthesis are shown the numbers of root hair measured per each sample.

in contrast to the variation of auxin levels where the relative difference in RH lengths between wt and atpgp4 was only slightly affected. Keeping the fact in mind that auxin application itself does not cause ectopic RH development [21,24] our data on auxin and ethylene treatments suggest that AtPGP4 is more likely to be involved in auxin- than in ethylene-dependent RH development.

3.4. *atpgp4 mutants reveal elevated auxin levels and auxin transport deficiencies*

Auxin promotes LR initiation [16,25] and RH elongation [21,22] while enhanced LR formation and RH lengths in *atpgp4* plants can be mimicked by exogenous auxin. This prompted us to investigate free auxin levels in the mutant root. GC–MS analysis indeed showed elevated free IAA in the root of both mutant alleles of atpgp4 (224%, Fig. 4) while in the shoot differences were not significantly different (*atpgp4-1*) or only slightly different (*atpgp4-2*).

Auxin-related growth phenotypes and elevated auxin concentrations in the root suggested transport deficiencies in the root. Therefore, we measured in vivo ^{3}H-IAA uptake into wt and atpgp4 roots. Surprisingly, both mutant alleles showed significantly reduced IAA uptake compared to the wt (Fig. 5A).

In order to verify these data, a novel self-referencing microelectrode [33] was used. In short, this electrode allows

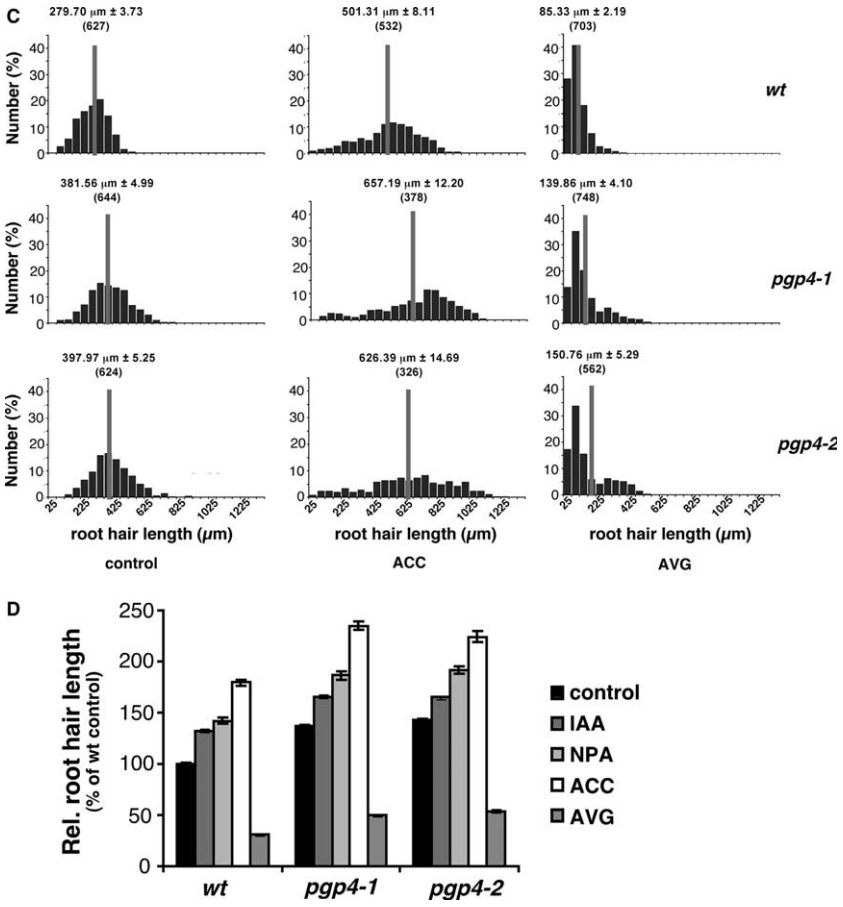

Fig. 3 (*continued*)

non-invasive and continuous recordings of auxin fluxes in intact root apices and has been shown to be highly selective for IAA. Characteristically, IAA influxes showed a distinct peak at 0.2–0.3 mm from the root apex in the so-called transition zone (TZ) or distal elongation zone of the root apex. In wt roots this transition zone peak averaged 193 ± 16 fmol cm^{-2} s^{-1}, while in atpgp4-1 the maximum influx in the TZ was significantly reduced averaging 133 ± 13 fmol cm^{-2} s^{-1} (Fig. 5B). At more distal positions, corresponding to cells accomplishing their onset into rapid cell elongation (>0.5 mm), the IAA influx was smaller and remained unchanged at positions as far back as 1 mm from the root apex. AtPGP4 roots showed also a reduced response to the inhibitory effect of NPA on IAA transport. In wt roots, the IAA influxes measured in the TZ of the root apex after 2 h of NPA treatment (20 μM) were about 100 ± 5 fmol cm^{-2} s^{-1} (around 50% inhibition), whereas in atpgp4-1 roots the inhibitory effect of NPA was just 25% (133 ± 13 fmol cm^{-2} s^{-1} against 98 ± 10 fmol cm^{-2} s^{-1}). Interestingly, reduced sensitivity of IAA influx toward NPA in the atpgp4 TZ are in accordance with the reduced effect of NPA on LR and RH formation in atpgp4 mutants.

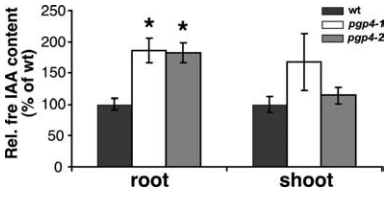

Fig. 4. Roots of atpgp4 plants show elevated levels of free IAA. Root and shoot segments were methanol extracted and free IAA analyzed by GC–MS. Shown are mean numbers of two independent experiments (each 30–50 seedlings) ± S.E., significant differences to wt growth (*P* values analyzed by Student's *t* test) are marked with an asterisk.

In order to investigate at the cellular level if AtPGP4 itself is the component responsible for IAA transport, we functionally expressed AtPGP4 in yeast. Fluorinated indolic derivates, toxic analogs of potential IAA precursors, are cytotoxic to yeast and have become a valuable tool used to investigate auxin transport [34]. Using yeast strain JK93da that lacks endogenous PGP-like proteins [7] we found that AtPGP4 provides hypersensitivity toward 5-fluro-indol compared to the vector control (Fig. 5C, left panel). In order to verify these data, we repeated growth analysis using the IAA-sensitive mutant yeast strain *yap-1-1*. In short, this yeast strain is sensitive toward low concentrations of IAA due to transcriptional upregulation of endogenous AUX1-like IAA uptake systems [31] and has therefore become an excellent system to demonstrate auxin transport [11]. Again, AtPGP4 confers slight but significant hypersensitivity to yap1-1 yeast (Fig. 5B, right panel). AtPGP4 expression is under control of a GAL promoter; the fact that hypersensitivity is dependent on galactose in the growth medium (results not shown) excludes secondary effects. Unlike AtPGP1, which specifically complemented growth deficiencies on 5-fluoro-indol and IAA thus demonstrating IAA export [11] both growth tests argue for an import direction. In summary, both root transport and yeast complementation data

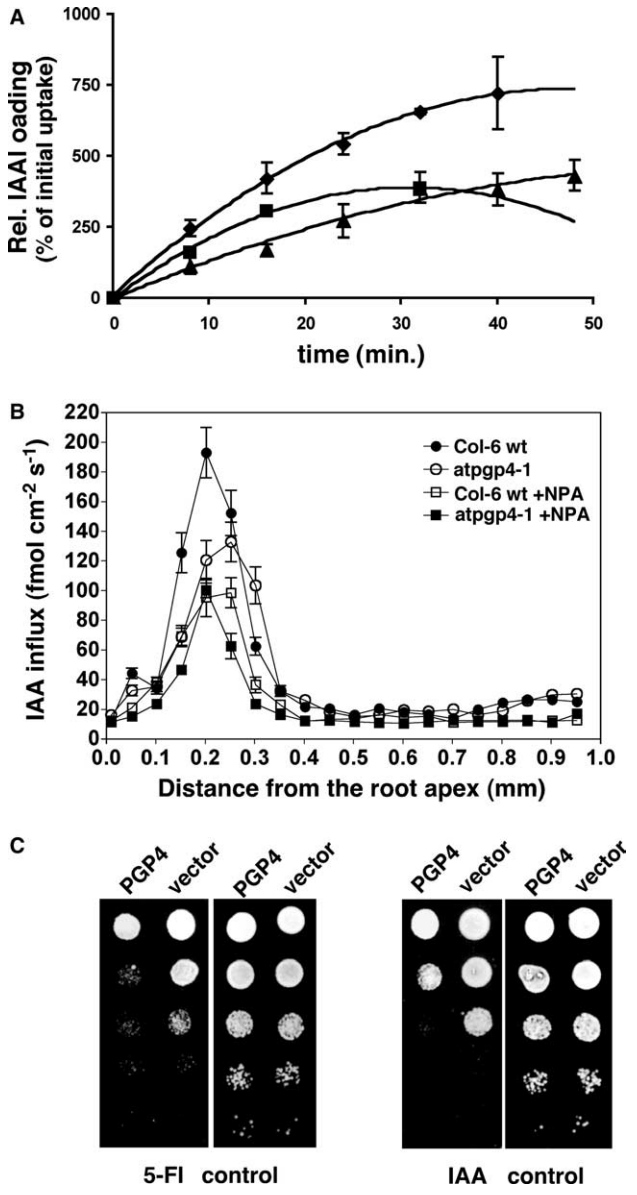

Fig. 5. Auxin transport is altered in *atpgp4* plants. (A) Roots of atpgp4-1 (filled square) and of atpgp4-2 (filled triangle) show reduced 3H-IAA uptake compared to the wild-type (filled circle). (B) Diagram illustrating the IAA influx profile along single roots of wt and atpgp4-1 plants and the effect of NPA (20 μM). Differential current from an IAA-selective microelectrode placed 2 μm from the root surface and used in a self-referencing mode. The sensor was vibrated between two positions 10 μm distant at a rate of 0.1 Hz. Positive fluxes represent a net IAA influx. Data shown were collected continuously over a 10 min period and are means of eight replicates. Error bars represent S.E. (C) AtPGP4 confers hypersensitivity to IAA sensitive *yap1-1* mutant strain. 10-fold dilutions of yeast cells transformed with AtPGP4 or the corresponding vector control were spotted on control plates or plates supplemented with 10 μM IAA.

point to an in vivo uptake of IAA over the plasma membrane. Uptake by an ABC MDR-like ABC transporter is an unusual event and has to our knowledge only been demonstrated once: CjMDR1 from *Coptis japonica* has been shown to catalyze the primary active uptake of the alkaloid berberine [27]. Interestingly, AtPGP4 shares (beside to its closest homologue AtPGP21) highest homology with CjMDR1 (65%) and in a phylogenetic tree AtPGP4/AtPGP21 and CjMDR1 cluster together on a subcluster that is clearly distinct from the one that is characterized by AtPGP1 and AtPGP19 (results not shown). This indicates that in analogy to CjMDR1, AtPGP4 might catalyze the cellular import of auxin.

4. Conclusions

Using reverse genetics we provide several lines of evidence suggesting that AtPGP4 is involved in auxin-mediated LR and RH development: (i) A survey of available microarray data and RT-PCR verification demonstrate expression in early root development. (ii) atpgp4 plants show enhanced LR initiation and RH elongation both known to be controlled by auxin. (iii) Exogenous IAA and auxin transport inhibitor, NPA, can mimic both mutant phenotypes when applied to the wt but wt and atpgp4 plants show different sensitivities toward IAA and NPA. (iv) AtPGP4 loss-of-function mutants reveal elevated root free auxin levels.

The reduced root IAA uptake capacities shown by transport studies and an IAA-selective electrode as well as the fact that AtPGP4 confers hypersensitivity to the IAA sensitive yap1-1 yeast mutant suggest an involvement of AtPGP4 in auxin transport like previously shown for AtPGP1 [11]. Surprisingly, all experimental setups suggest an uptake direction, which has been demonstrated only once for the closely related CjMDR1 [27]. Elevated root auxin levels might account for the described auxin-related growth phenotypes but seem on a first view to be contradictory with the proposed transport direction. The simplest model to explain growth phenotypes, elevated auxin concentration and a putative involvement in root auxin uptake would be to postulate an involvement of AtPGP4 in polar auxin transport. In this so far speculative model, AtPGP4 would co-function with AtPGP1 in basipetal auxin transport from the root to the shoot. AtPGP4 and AtPGP1 would catalyze active (ATP-dependent) auxin import and export, respectively, at opposite (apical and basal, respectively [36]) plasma membrane domains of the cell. Obviously, this would require a polar expression pattern of AtPGP4 but so far the precise location of AtPGP4 is unknown.

However, a putative polar expression and involvement in PAT, like found recently for AtPGP1 in the root elongation zone [11], is supported by two findings. First, LR formation in atpgp4 mutant plants is less affected than in wt plants by NPA, known to block basipetal IAA reflux from the root tip [16]; NPA inhibition [11] and binding [12] was shown for AtPGP1. Second, AtPGP4 has been identified in a proteomic inventory to reside on detergent-resistant plasma membranes (DRMs) or lipid rafts [28]. In plant cells, these microdomains are thought to play an important role in protein targeting and polar distribution of plasma membrane proteins, including putative auxin facilitator PIN1 [28,29].

Acknowledgments: This work was financially supported by the Swiss National Science Foundation (E.M.). We thank Drs. M. Lopes for careful reading of the manuscript and A. Murphy for providing AtPGP4 cDNA (pBSKII-PGP4).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.08.061.

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3.6 Flavonoids redirect PIN-mediated polar auxin fluxes during gravitropic responses

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the journal of biological chemistry

In revision by the Journal of Biological Chemistry, minor revisions asked

Here, we investigated the role of flavonoids in root gravitropism by comparing the gravitropic responses of *pin2* - an agravitropic auxin transport deficient mutant - with the corresponding wild type plants. By means of an HPLC-ESI-MS/MS approach and DPBA fluorescences studies, we were able to demonstrate that *pin2* mutants have impaired flavonoid glycosides accumulation patterns, with a shift from di- and triglycosylated flavonols to monoglycosylated flavonols. Surprisingly, we found that application of exogenous flavonoids at low concentration (100 nM) could partially rescue *pin2* agravitropic phenotypes, by triggering the formation of lateral auxin gradients in a PIN2-indipendent manner. Chemical complementation by flavonoids strictly correlated with an asymmetric distribution of the PIN1 protein across the root tip of *pin2* mutants, suggesting that PIN1 is the auxin efflux carrier that facilitates basipetal auxin fluxes for gravitropic responses in flavonoid-treated *pin2* roots.

Our work uncovered for the first time a link between defects in polar auxin transport and deficiencies in flavonoid glycosides accumulation in the root elongation zone and suggests that flavonoids might be positive regulators of PIN1 activity.

Title page

Flavonoids Redirect PIN-mediated Polar Auxin Fluxes during Root Gravitropic Responses

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Running title: Redirection of auxin flows by flavonoids

Key words: Flavonoid, PIN2, root gravitropism, polar auxin transport, PIN1, quercetin

Summary

The rate, polarity and symmetry of the flow of the plant hormone auxin are determined by the polar cellular localization of PIN-FORMED (PIN) auxin efflux carriers. Flavonoids, a class of secondary plant metabolites, have been suspected to modulate auxin transport and tropic responses. Nevertheless the identity of specific flavonoid compounds involved and their molecular function and targets *in vivo* are essentially unknown.

Here we show that the root elongation zone of agravitropic *pin2/eir1/wav6/agr1* has an altered pattern and amount of flavonol glycosides. Application of non-inhibitory concentrations of flavonols to *pin2* roots is sufficient to restore root gravitropism. By employing a quantitative cell-biological approach, we demonstrate that flavonoids restore the formation of lateral auxin gradients in the absence of PIN2. Chemical complementation by flavonoids strictly correlates with an asymmetric distribution of the PIN1 protein. *Pin2* complementation does not result from inhibition of auxin efflux, as supply of the auxin transport inhibitor *N*-1-naphtylphtalamic acid failed *per se* to restore *pin2* gravitropism.

We propose that flavonoids promote asymmetric PIN shifts upon gravity stimulation, thus redirecting basipetal auxin streams necessary for root bending.

Introduction

The plant hormone auxin (3-indolyl acetic acid, IAA) controls virtually all plant developmental and physiological processes. In roots, the differential growth response associated with gravity stimulation (gravitropism) occurs in the elongation zone (1,2) and is a result of the asymmetric distribution of auxin to the lower side of epidermal cells (3). In these tissues accumulating auxin, cell elongation is inhibited and the root tip bends downwards. This cell-to-cell or polar auxin transport (PAT) is determined by the asymmetric cellular localization of auxin in- and efflux components of the PGP/MDR/ABCB, AUX1/LAX and PIN-FORMED (PIN) family (4-7). While PGPs are apparently more involved in long-range auxin transport (8-10), AUX1 and PIN2/*EIR1/WAV6AGR1* channel auxin from the lateral root cap basipetally to the expanding epidermal cells (11-13).

The regulation of auxin transport during root gravitropic responses is still largely unclear. Among various possible mechanisms, the localized synthesis and directed transport of flavonoids, plant-specific phenylpropanoid compounds, have been shown to modulate the rate of the gravity response (14,15). A number of lines of experimentation have suggested that flavonoids may act as non-essential auxin transport inhibitors (16-20). This is mainly based on the finding that flavonoids displace binding of synthetic auxin transport inhibitors, like 1-*N*-naphtylphtalamic acid (NPA), *in vitro* (Jacobs and Rubery, 1988; Lomax et al., 1995; Luschnig, 2001; Morris, 2000). Moreover, roots of *transparent testa* (*tt*) Arabidopsis mutant with manipulated flavonoid levels, exhibit altered gravitropic curvature and auxin transport, which are restored to wild-type level by exogenous application of flavonoids (16,21). Nonetheless, the identity of the specific flavonoid compounds involved, their molecular targets as well as their mode of action *in vivo* are essentially unknown. While several lines of evidence suggest that PGPs are modulated by aglycone flavonols (8-10,17,22), the expression and sub-cellular location of PIN auxin efflux carriers is thought to be a consequence of flavonoid-mediated alteration of auxin concentrations (19,20).

Here, we report that agravitropic loss-of-function mutant *pin2/eir1/wav6/agr1* has impaired patterns of flavonol glycosides. We found that nM concentrations of exogenous flavonols, which have no

inhibitory effect on root elongation and gravitropic response in wild-type plants, can rescue the agravitropic phenotype of *pin2* roots by promoting asymmetric PIN1 shifts, re-establishing polar auxin fluxes. This is the first report that mechanistically links flavonoid action to polar auxin transport, suggesting that the role of flavonoids is not restricted to inhibition of efflux transporters (17,22-24), but that they are able to function as versatile modulators of polar auxin flows establishing the basis for physiological flexibility.

Experimental procedures

Chemicals

MeCN (HPLC Supra grade, Scharlau, E-Barcelona), HCOOH (Fluka, puriss, Switzerland), Methanol (MEOH, Fisher Scientific, UK), HPLC-grade acetonitrile (Fisher Scientific, UK), H_3PO_4 (Applichem, Germany). Water was purified with a MilliQ Gradient apparatus (<5ppb, Millipore, Milford, MA, USA). Diphenilyboric acid 2-aminoethyl ester (DPBA, Sigma, Germany). NPA, (Fluka, Germany), kaempferol (CalbioChem, La Jolla, CA) and quercetin (Fluka, Germany) were dissolved in 100% dimethyl sulfoxide (DMSO).

Growth conditions and plant material

Seeds were surface sterilized for 5h in a chamber containing vaporous HCl and Na-Hypochlorite and stratified in a 0.1% agar solution for 2d at 4°C. Subsequently, the seeds were plated on sterile half-strength MS medium at pH 5.7 containing 2% sucrose solidified with 0.6% phytagel (Sigma, Buchs, Switzerland), and vertically grown at 22°C with 16h/8h light/dark cycle. The mutant alleles used in this study are the following: *pin2-1* (25) and *eir1-4* (26).

Flavonoid fluorescence staining

Flavonoid compound locations were visualized *in vivo* by the fluorescence of flavonoid-conjugated DPBA to the compounds after excitation with blue light. Plants were grown for 5d before staining. Fluorescent staining of whole seedlings was performed according to Buer and Muday (2004) (14). Fluorescence was achieved by excitation with FITC filters (450 to 490nm, suppression long pass 515nm) on a Leica DMR fluorescence microscope and a 10X or 20X objective. Digital images were captured with a Leica DC300 F charge coupled device (CCD) camera.

Extraction of phenolic compounds and HPLC analysis

Excised roots were incubated over night in the dark at 4°C in 0.5ml of 80% (v/v) methanol (MeOH), extracted and centrifuged at 18'000g for 10min. The supernatant was concentrated to dryness and resuspended in 0.1ml 80% MeOH. Aliquots (50 ml) were analyzed by a reverse-phase HPLC (Gynkotek, Germany). Absorbance spectra were recorded with a UVD340S diode array detector (Dionex, Switzerland). Data integration analysis was conducted using the Chromeoleon software (v6.4, Dionex, Switzerland). The peak height was quantified at 330 nm. A calibration curve for kaempferol was used as reference for single peak quantification. All analyses were performed with at least three independent replicates, each representing 100 roots. Chromatographic conditions: Nucleosil 100-5 C_{18} column (5µm, 2 x 250 mm, Macherey-Nagel, Düren, Germany); flow rate 1.00ml min^{-1}, gradient (step, time, %B over A) 1, 25min, 10-25%; 2, 10min, 25-70%). Solvent A: H_2O/0.1% (v/v) H_3PO_4; solvent B: MeCN.

Structural elucidation: HPLC-ESI-MS/MS analysis

HPLC-MS analyses were performed on an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, U.S.A.) fitted with a HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland), an Agilent 1100 binary pump, and an Agilent 1100 photodiode-array detector. Chromatographic conditions: Nucleosil 100-3 C_{18} column (3µm, 2 x 250mm, Macherey-Nagel, Hoerdt, France); flow rate

0.170ml min^{-1}, gradient (step, time, %B over A) 1, 25min, 10-25%; 2, 10min, 25-70%). Solvent A: H_2O/0.1% (v/v) HCOOH; solvent B: MeCN/0.1% (v/v) HCOOH. The HPLC was connected to a Bruker ESQUIRE-LC quadrupole ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany), equipped with a combined Hewlett-Packard Atmospheric Pressure Ion (API) source (Hewlett-Packard Co., Palo Alto, CA, USA). The HPLC output was directly interfaced to the ESI ion source. The MS-conditions were: Nebulizer gas (N_2) 40psi, dry gas (N_2) 9 l/min, dry temperature 300°C, HV capillary 4000V, HV EndPlate offset –500 V, capillary exit –100V, skimmer1 –28.9 V, and trap drive 53.4. The MS acquisitions were performed in the negative electro spray ionization mode, at normal resolution (0.6u at half peak height), under ion charge control (ICC) conditions (10'000) in the mass range from *m/z* 100 to 1000.

The MS^2 acquisitions were obtained in the auto-MS/MS mode. The isolation width was 4u, the fragmentation cut-off set by "fast calc", and the fragmentation amplitude set at 0.9V in the "SmartFrag" mode. The total amounts of flavonoid compounds were calculated as the sum of the areas ($x10^6$ AU, arbitrary unit) of the mass signals identified during HPLC-ESI-MS/MS analysis. Each extraction consists of a pull of 100 different roots. Quantification of RT-EZ flavonoid compounds is the result of two independent extractions in which each time 150 5-mm long root apices from 12 different agar plates were pulled together.

Gravitropic assays

4d light-grown seedlings were transferred from control plates to plates containing nutrient media optionally supplemented with quercetin or kaempferol (100nM or 200nM). After 24h of adaptation and growth in the new media, plates were turned 90°. In one type of gravitropic assay, after additional 24h of growth under gravistimulation, seedlings were scanned using Epson Perfection 2450 photo and angles of gravitropic curvature were measured from digital pictures using the tool "Image Manager" of the Leica IM1000 software (Leica, Heerbrug, CH). Each gravity stimulated root was assigned to one of twelve 30° sectors; the length of each bar represents the percentage of seedlings showing the same direction of root tip growth. Short pulses of gravity stimulation were achieved by turning the plates of 90° for 2h, which corresponds to the peak of gravity-induced flavonoid accumulation (14). After 2h of gravity stimulation, roots were excised and flavonoids extracted as described, or expression of $DR5_{rev}$-GFP reporter protein analysed on a Leica TCS SP2 CLSM. All gravitropic assays were performed in the dark to prevent phototropic responses. In some cases, angles of gravitropic curvature were measured in a blind assay, to reduce possible unbiased calculations.

Immunocytochemistry

PIN1 immunolocalization was performed as previously described (27) with PIN1 specific antibody (28) at 1:1000 dilution and anti-rabbit CY3 conjugated secondary antibodies. Confocal imaging of CY3 and *DR5rev::GFP* was carried out on a Leica SP2 AOBS microscope.

Data analyses

Statistical analysis was performed using SPSS 11.0 (SPSS Inc., Chicago, Illinois).

Results

Pin2 roots have an altered flavonoid pattern

Pin2/eir1/wav6/agr1 Arabidopsis mutant (referred to as *pin2* hereafter), one of the best-characterized auxin transport mutants, exhibits reduced basipetal auxin transport and agravitropic root growth (2,25,26,29). As a starting point of this work, we investigated whether defects in basipetal auxin transport in *pin2*, which result in agravitropic responses (30), are linked to an altered accumulation of specific

endogenous flavonoids and whether flavonoids could be directly implicated in the control of the gravitropic responses. Diphenylboric acid 2-amino-ethylester (DPBA), a fluorescent dye that specifically interacts with flavonoids, allows *in situ* flavonoid staining and localization in Arabidopsis seedlings (19,31,32). In wild-type seedlings, flavonoid DPBA staining is restricted to the shoot apex and cotyledons, the root-shoot junction, along the primary root, and most intensely to the root elongation zone (Figure 1A and Figures S1A-C) (31). In contrast, flavonoid-DPBA fluorescence in *pin2* mutant was clearly lower at the root tip-elongation zone (RT-EZ) (Figure 1B and Figures S1E-G). Manipulation of endogenous auxin levels by addition of 100nM IAA increased DPBA fluorescence in the wild type (19) and, although to a lesser extend, also in the *pin2* RT-EZ (Figures S1D and S1H), suggesting that auxin and flavonoid levels *in planta* are interconnected (19,32).

To determine how flavonoid distribution was affected by PAT alterations, we qualitatively and quantitatively investigated endogenous flavonoid derivatives present in wild-type and *pin2* RT-EZ and entire roots using HPLC-UV-(-)-ESI-MS/MS and HPLC-ESI-MS, respectively (Table I). Consistent with DPBA staining profiles (Figures 1A-B and S1), we found that the total amount of flavonoids was significantly reduced in the RT-EZ of *pin2* mutant (Figures 1C and 1D) whereas no significant difference was observed over the entire root (Figures 1C and S2). *Pin2* roots showed altered accumulation of specific flavonol glycosides both in the RT-EZ and in the entire root (Table I and arrows in the extraction ion chromatograms (EIC) of the masses of interest in Figures 1D and S2). In *pin2* entire roots and RT-EZ, a shift from di- and triglycosylated flavonols to monoglycosylated flavonols, like for K-G-3 (compound **18**), was observed (Table I). Intriguingly, accumulation of compounds glycosylated in the 7-*O*-position was strongly reduced in *pin2* (Table I), which suggests that auxin levels may have an effect on the corresponding glycosyltransferases. Conversely, those peaks, whose accumulation is affected in *pin2* roots, may be functionally important for the regulation of auxin transport during root gravitropism.

As previously reported (14,33), gravity stimulation increased the DBPA fluorescence in wild type RT-EZ by nearly two-fold, with a maximum at 1.5 to 2.5h after stimulation. A smaller but significant increase in DPBA fluorescence was observed also in *pin2* mutant (Figures 1E-F). Flavonoid quantification by HPLC-UV (Figure 1G) was consistent with the DPBA staining (Figures 1E-F).

Collectively, our results demonstrate that the synthesis and the transient accumulation of specific flavonoid glycosides in the root tip-elongation zone - but not over the entire root or in the shoot - are impaired quantitatively and qualitatively in *pin2* (Figures 1, S1, S2).

Flavonoids rescue the agravitropic response of *pin2* roots

To test whether flavonoid concentrations play a critical role in the response to gravity stimuli, we searched for conditions in which flavonoids could be supplied without affecting root growth and gravitropism by acting as auxin transport inhibitors (16,19). Concentrations up to 100 nM kaempferol or quercetin did not significantly influence wild-type gravitropic responses (95.5% and 99.0% instead of 99.4% (sum of 90° and 120° sectors), Figure 2A). Roots of the *eir1-4* mutant, a severe agravitropic allele of *pin2* (26), were gravity-stimulated in the presence of 100 nM flavonoids. Intriguingly, *pin2* gravitropic root bending was partially restored by quercetin (50.0%) and kaempferol (52.1%, Figure 2A). The same gravitropic assay was performed in the presence of 5µM 1-*N*-naphtylphtalamic acid (NPA), a synthetic polar auxin efflux inhibitor and herbicide (naptalam©), blocking basipetal IAA movement from the

root tip (34). In wild type plants, NPA treatment resulted in an agravitropic phenotype (39.4%) (16,30). However, NPA failed to restore but rather impaired *pin2* root gravitropism (14.7%). We repeated the quercetin treatment in the presence of NPA, and found that in wild type NPA and quercetin had additive effect (28.6%), while in *pin2* the quercetin action was prevented by NPA (13.0%), indicating that the rescue of *pin2* agravitropic response by quercetin is NPA sensitive.

The cause of *pin2* agravitropic root growth is a failure in the accumulation of auxin at the lower side of the root elongation zone (26). We therefore tested whether flavonoid treatment restored the asymmetric auxin distribution necessary for differential growth of the epidermal cells during gravitropic responses by monitoring the *DR5:GFP* expression, which reflects relative auxin levels. As previously reported (35), in wild type seedlings with vertically grown roots, the GFP fluorescence appeared in specific stele cell files and was localized in the quiescent center (QC), in the columella initials (QI) and in the mature columella cells (col) (Figure 2B). Vertically oriented *pin2* roots exhibited a strong signal in the distal lateral root cap (dLRC) with only weak extension towards the proximal lateral root cap (pLRC), reflecting defect in basipetal auxin distribution. In vertically oriented roots treatments with 100nM quercetin or kaempferol resulted in fluorescence essentially similar to the control condition in both the wild type and *pin2* mutant (Figure 2B). Upon gravity stimulation, fluorescent signals in wild-type roots appeared in the lower sides, in the distal lateral root cap and EZ, both in solvent controls (control) and, although slightly reduced, in the presence of flavonoids (Figure 2B, asterisks). In contrast, flavonoid treatment of gravity stimulated *pin2* roots resulted in a gain of a strong asymmetric signal in the entire lateral root cap with significantly increased fluorescence on the lower half of the root and decreased fluorescence on the upper half of the root (Figure 2B, asterisks).

In order to statistically quantify auxin accumulation after gravity stimulation, we determined auxin gradient intensities (in arbitrary units from 0 to 3) in root tips differently oriented relative to the gravity stimulation vector (for experimental details see legends of Table 2 and S1). Compared to wild type (11.7%, Table 2), the majority of *pin2* seedlings tested exhibited a symmetrical auxin distribution (53.8% of auxin gradient intensity 0). Only occasionally clear auxin gradients (defined as sum of 2 + 3) could be observed in *pin2* upon gravity stimulation (27.7% (2+3), Table 2), which is in line with the results of the gravitropic assays (Figure 2A). Interestingly, a similar frequency of clear auxin gradients in *pin2* was also observed in vertically grown roots (31.7%) but not found in the wild type (1.7%, Table S1). The gain of signal asymmetry between lower and upper side in flavonoid-treated, gravity stimulated *pin2* roots continued in the elongation zone and was observed in the majority of the flavonoid treated roots tested (62.0% compared to 27.7% without treatment, Table 2). In contrary, quercetin treatment had only low effect on wild type (51.4% compared to 46.7% without treatment). These data show that exogenous flavonoids are able to re-establish the asymmetric *DR5-GFP* activity in the *pin2* mutant roots and restore their gravitropic responses.

Flavonoids promote asymmetric PIN1 shifts

Since some levels of functional redundancy between PIN proteins have been already demonstrated (36,37), we tested the root gravitropic responses of the triple *pin2 pin3 pin7* mutant in the presence of 100nM quercetin. A strong agravitropic root phenotype was evident for *pin2 pin3 pin7* in control conditions (Figure 3A) (37), which could be partially rescued by the application of 100 nM of quercetin (47.8%

compared to 26.1% without treatment, Figure 3A) or kaempferol (data not shown). From this result, we conclude that neither PIN3 nor PIN7 are required for flavonoid-dependent rescue of the agravitropic response of *pin2*, being in-line with their proposed role and expression in gravity perception tissues (37). Importantly, when we performed the same gravitropic assay with roots of the double mutant *pin1 pin2*, quercetin could not complement its agravitropic phenotype (15.7% compared to 12.2% without treatment, Figure 3A). These data demonstrate that PIN1 is essential for flavonoid-dependent complementation of *pin2* gravitropism, which is in agreement with its expression in gravity transduction or response tissues.

To trace the behaviour of PIN1 protein during *pin2* gravitropic responses and to uncover a possible link between the action of flavonoids and PIN1 activity *in vivo*, we analysed PIN1 localization in *pin2* roots exposed to 100nM quercetin prior to, and during gravity stimulation. Consistent with previous reports (36,37), in vertically oriented wild type roots PIN1 was mainly found at the basal (lower) end of vascular and endodermis cells with occasional weak expression in the epidermis and in the cortex (Figure 3B, i.). In vertical *pin2* roots, PIN1 was ectopically expressed in the endogenous PIN2 domain, showing symmetric apical (up) localization in the epidermis and basal (down) localization in cortex cells (arrows in Figure 3B, ii.) (36). This symmetric PIN1 location was only rarely altered by a gravity stimulus on solvent control (12.5% of roots showing this pattern, Figure 3C, i.). Treatments with 100nM quercetin did not affect PIN1 expression or its symmetry neither in wild type nor in *pin2* vertically grown roots (Figures 3B, iii.- iv.). In contrast, gravity stimulation of *pin2* roots in the presence of 100nM quercetin resulted in asymmetric expression of PIN1 protein, with stronger PIN1-specific signals at the lower side of the root tip (Figure 3C, iii.). The establishment of flavonoid-mediated PIN1 gradients strictly correlated with the development of asymmetric DR5-GFP signals (100%, Fig. 3C, iii), while in the absence of quercetin asymmetric PIN1 patterns correlating with asymmetric DR5-GFP signals were only rarely found (12.5%, Fig. 3C,i). We never found asymmetric DR5-GFP gradients that correlated with symmetric PIN1 patterns (0%, Figure 3C, iv.) or symmetric DR5-GFP gradients correlating with asymmetric PIN1 expression (0%, Figure S3, i.). But for few roots we found a weak correlation between asymmetric PIN1 patterns and symmetric DR5-GFP (22.2%, Figure S3, iii.).

Discussion

Current paradigm in auxin research is that flavonoids act as non-essential, endogenous modulators of PAT by transiently accumulating in the epidermal cells of the root elongation zone (14,16,18-20,31). Our data demonstrate that defects in basipetal auxin transport are associated with altered root flavonoid accumulation. In conditions in which PIN2-dependent auxin transport is genetically blocked, synthesis and transient accumulation of specific flavonoid glycosides in the root tip-elongation zone - but not over the entire root or in the shoot - are impaired quantitatively and qualitatively (Figures 1, S1-S2). However, *pin2* roots retain the ability to accumulate flavonoids in response to a gravity stimulus, but not as efficiently as the wild type.

Application of low concentrations of exogenous flavonoids restores gravitropic root tip bending in a genetic background defective in basipetal auxin transport and this mode of flavonoid action is distinct from that of NPA. Moreover, our results showing that gravity stimulation of *pin2* roots in the presence of inhibitory concentrations of NPA did not complement *pin2* gravitropism, suggest that in our experimental conditions flavonoids do not act as PAT inhibitors. Quantitative cell biology analysis of DR5-GFP signals show that chemical complementation of

gravitropism by exogenous flavonoids is accompanied by re-establishment of asymmetric distribution of *DR5-GFP* in the *pin2* mutant roots. This suggests that restoration of gravitropic responses is achieved via bypassing the requirement of an active PIN2 protein, thus implying the activation of a PIN2-independent mechanism for basipetal auxin transport.

Quantification of root gravitropic response of different *pin* mutant combinations suggests that PIN1 is essential for flavonoid-dependent complementation of *pin2* gravitropism, which is in agreement with its expression in gravity transduction or response tissues. Using a quantitative cell biological approach, we also provide evidence that flavonoid-dependent rescue of *pin2* agravitropism by PIN1 is correlated with asymmetric PIN1 distribution across gravity-stimulated tissues. In summary, our findings suggest that PIN1 is the auxin efflux complex component that facilitates basipetal auxin fluxes for gravitropic responses in flavonoid-treated *pin2* roots. The observed basal-apical PIN1 shifts in *pin2* roots (Figure 3B, ii) are in line with the finding that PIN1 and PIN2 have redundant roles in the root meristem size control (37) and that PIN1 can functionally replace PIN2 when ectopically expressed and localized at the upper side of epidermal cells (5). Moreover, PIN1 showed a "PIN2-like" apical localization in epidermis and basal localization in cortex cells in roots of *pin2* mutants (36). However, our data indicate that flavonoids are the native key effectors that promote asymmetric PIN1 shifts with stronger PIN1-specific signals at the lower side of the root tip in response to a gravity stimulus, thus redirecting basipetal auxin streams necessary for the root tip bending.

The fact that flavonols can inhibit PAT and displace NPA from their membrane binding sites has led to the idea that flavonoids and NPA act on similar targets using identical mechanisms. While several lines of evidence suggest that plant PGPs, like PGP1, PGP4 and PGP19, are direct targets of flavonoid regulation (8,17,22) either via protein phosphorylation, inhibition of ATPase activity or allosteric binding in analogy to mammalian PGPs (38), the effect of flavonoids on the members of the PIN family has received less attention and seems to be indirect (19,32). With this work we provide two lines of evidence demonstrating that flavonoids, at least under our experimental conditions, do not solely inhibit efflux transporters (in analogy to NPA) (17,22,23), but are able to function as versatile modulators of polar auxin flows. Firstly, flavonoid concentrations applied to the roots did not significantly alter wild type gravitropism and therefore most likely also not PAT. Secondly, NPA failed to restore *pin2* root gravitropism while rescue of *pin2* agravitropic response by quercetin was NPA sensitive. Our data showing that flavonoids can promote PIN1 shifts in response to a gravity stimulus underline an involvement of flavonoids in cellular trafficking of auxin transport complex components as recently suggested (19,32). The modulation of root gravitropism by flavonoids may result from a combination of PGP regulation and PIN trafficking that might be concentration-dependent and interconnected.

While the cellular targets of flavonoid action are now known, the underlying mechanism remains elusive. Transport assays with PIN proteins suggest that flavonoids probably do not interact with PINs directly. However, flavonoids affect specific PIN expression, location and cellular trafficking probably through interaction with regulatory proteins (19,32). Recently, plasma membrane PIN shifts have been demonstrated to be caused by antagonistic PIN phosphorylation via protein kinase PINOID (PID) and protein phosphatase 2A (PP2A) (39,40). Our data together with the fact that flavonols are routinely used as both protein phosphatase and kinase inhibitors make PID or PID-related WAG kinases (41) and/or PP2A-like phosphatases the most likely candidate targets for flavonol-mediated PIN-shifts

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Footnotes

We would like to thank Dr. Heller (GSF, Munich) and Dr. Veit (Kaufering) for providing us with flavonol standards that were invaluable for the flavonol structure analysis and V. Sovero and S. Peters for comments on the manuscript. This work was supported by funds of the Swiss National Foundation to M.G. (3100AO-111912) and to E.M. (within the NCCR Plant Survival)), the Novartis foundation (to M.G.) and by the Ministry of Science and Technology of South Korea (to Y.L. and E.M.).

Figure legends

Figure 1. Defects in basipetal auxin transport are associated with altered root flavonoid accumulation.

A-D, Flavonoid accumulation in the entire root and root elongation zone of wild type (Col Wt) and *pin2* (A, wild type; B, *pin2*). EZ, elongation zone; RT, root tip. Bar, 100µm. (C) Total amount of flavonoid derivatives detected in the entire root and RT-EZ of wild type and *pin2*. Values represent means ± SE (*n*=2-5 replicates); * significantly different from the wild type (Student's *t*-test, $P<0.05$).

D, Representative sum of extracted ion chromatograms $[M-H]^-$ of flavonoid derivatives found in wild type and *pin2* RT-EZ analyzed by HPLC-ESI-MS. Significantly altered compounds are indicated by arrows. Peak numbers correspond to flavonoid derivates listed in Table I. Note 10-times lower intensitiy scale for *pin2* root elongation zone in comparison to wild type.

E-G, Accumulation of flavonoids in 2h gravity stimulated roots (E, wild type; F, *pin2*). The arrows indicate the direction of the gravity vector relative to the root. Bar, 100µm. *In situ* flavonoid visualization (A, B, E, F) using DPBA (yellow fluorescence) was performed as described in Materials and methods.

G, Gravity-induced root phenolic compound accumulation normalized to phenolic compound accumulation in vertical control. Values represent means ± SE (*n*=2-5 replicates); * significantly different from the wild type (Student's *t*-test, $P<0.05$).

Figure 2. Exogenous flavonols but not NPA partially rescue the agravitropic response of *pin2* by restoring asymmetric auxin gradients.

A, Gravity responses of wild type (Col Wt) and *pin2* (*eir1-4)* roots after reorientation of 90° to horizontal (see sketch). The length of each bar represents the mean percentages ± SE of seedlings showing the same direction of root growth of at least three independent experiments; numbers correspond to the sum of 90° and 120° sectors.

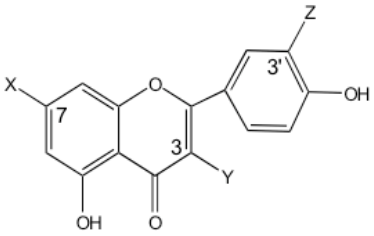
B, Expression of the auxin-reporter construct $DR5_{rev}$-GFP in wild type (Col Wt) and *pin2* (*eir1-4)* root tips was assessed prior to, and after 2h gravity stimulation on control (top row), quercetin (mid row) and kaempferol treated roots (bottom row). White asterisks indicate more pronounced *DR5-GFP* expression at the lower side of gravistimulated roots indicating enhanced basipetal auxin reflux. The gravity vector relative to the root tip is indicated by an arrow. QC, quiescent center; QI, columella initials; col, mature columella cells; dLRC, distal lateral root cap; pLRC, proximal lateral root cap. Bar, 75 µm.

Figure 3. Flavonoid-dependent rescue of *pin2* agravitropic phenotype requires PIN1 and is correlated with asymmetric PIN1 distribution across gravity-stimulated tissues.

A, Gravity responses of *pin2 pin3 pin7* and *pin1 pin2* (*pin1 eir1*) roots. The length of each bar represents the mean percentages ± SE of seedlings showing the same direction of root growth of at least three independent experiments; numbers correspond to the sum of 90° and 120° sectors.

B, Whole-mount *in situ* immunolocalization of PIN1 protein (red) in 5d *pin2* (*eir1-4*; ii. and iv.) and wild type (Col Wt; i. and iii.) vertically seedlings transferred on media supplemented with 100nM quercetin. Gravity vector is indicated by an arrow. White arrows indicate PIN1 protein apical localization in the epidermis and basal localization in the cortex cells of *pin2* root tip. Note that the appearance of slightly different PIN1 signals in the *pin2* epidermis and cortex (ii.-iv.) do not reflect unequal expression but are the result of unequal background intensities due to scattered light. v, vascular bundle; en, endodermis; c, cortex; e, epidermis. Bar, 30 µm.

C, Whole-mount *in situ* immunolocalization of PIN1 protein in *pin2* after 2h of gravity stimulation; gravity vector is indicated by an arrow. 4d *pin2* seedlings were transferred on media supplemented with 100nM quercetin or the solvent (control). Red, PIN1; green, $DR5_{rev}$-GFP expression. White arrows indicate more pronounced PIN1 proteins levels at the lower or upper side of gravity stimulated root tip. Bar, 30µm. Percentages indicate relative occurrence of asymmetric or symmetric PIN1 distributions with asymmetric DR5-GFP signals; the total number of analysed roots that showed simultaneous clear DR5-GFP and PIN1 signals was 47.

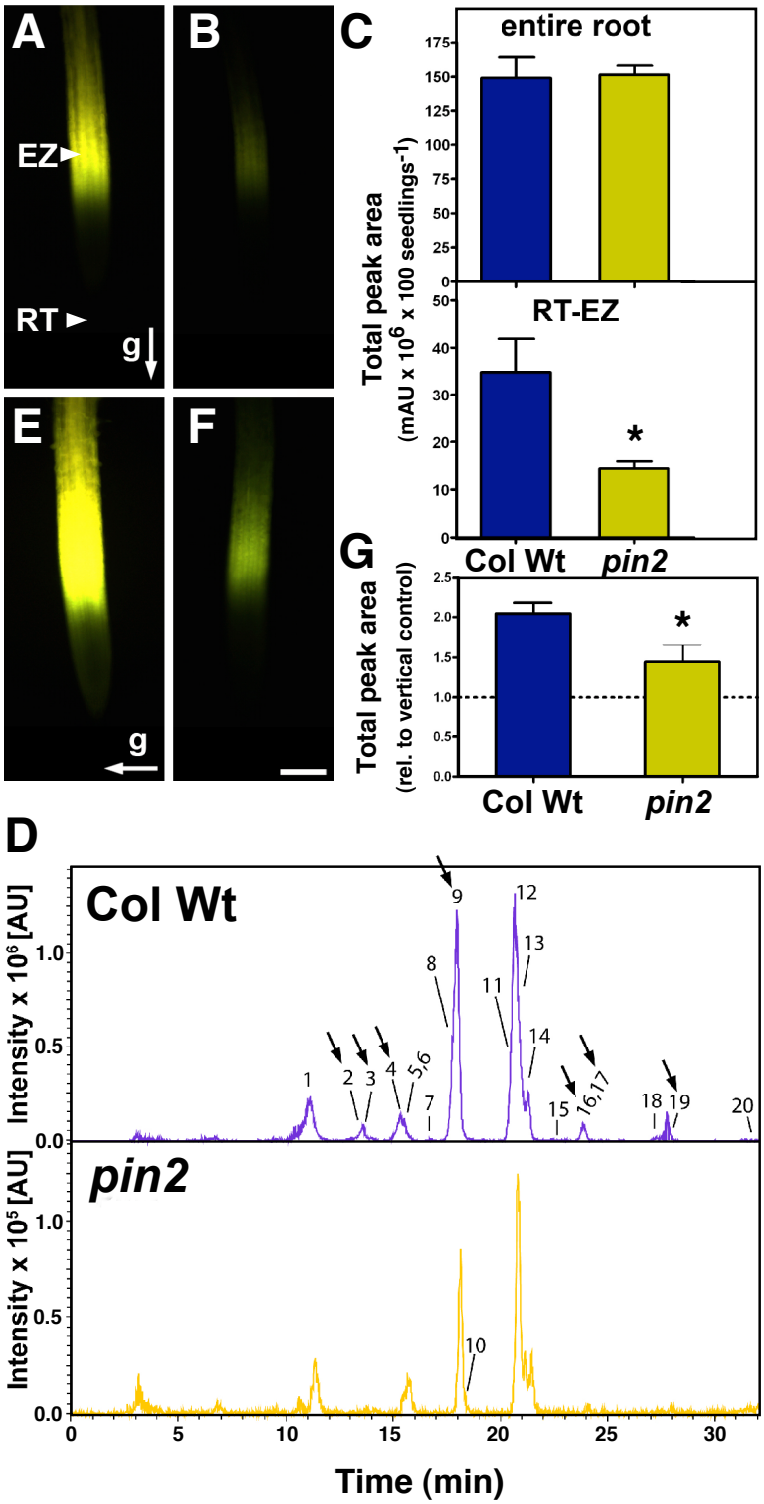


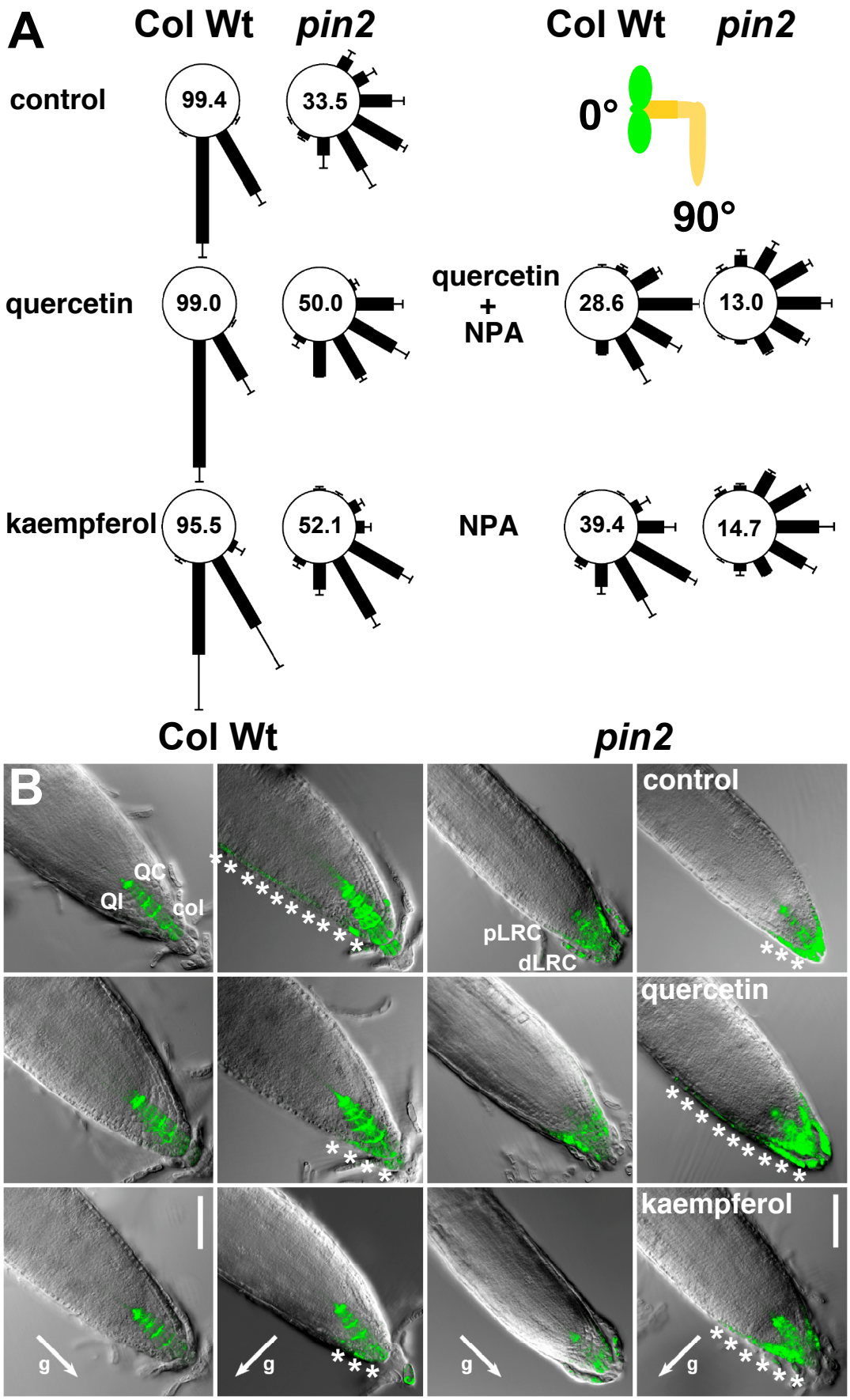
compound	Rt (min)	MW	trivial name	entire root (Area x 10^6 AU)		root tip (Area x 10^6 AU)	
				Col	*pin2*	Col	*pin2*
2	13.6	756	**Q-R-G-3-R-7**	5.9 ± 0.47	3.7 ± 0.4	1.25 ± 0.2	0.2 ± 0.1
3	14.2	610	**K-G-3-G-7**	1.7 ± 0.32	1.2 ± 0.3	0.2 ± 0.1	0.1 ± 0.0
4	15.5	740	**K-R-G-3-R-7**	10.1 ± 0.51	9.0 ± 0.7	1.3 ± 0.2	0.4 ± 0.1
5	15.8	772	**Q-G-G-3-R-7**	1.2 ± 0.05	1.5 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
7	16.8	594	**K-R-3-G-7**	0.7 ± 0.15	0.2 ± 0.03	0.1 ± 0.03	< 0.1
8	17.8	756	**Q-G-R-R**	2.3 ± 0.25	0.9 ± 0.2	0.3 ± 0.1	< 0.1
9	18.1	610	**Q-G-3-R-7**	52 ± 4.1	43.5 ± 1.0	15.6 ± 3.5	5.3 ± 0.7
10	18.3	756	**K-G-G-3-R-7**	n.d.	1.1 ± 0.2	n.d.	< 0.1
12	20.8	594	**K-G-3-R-7**	49.8 ± 7.7[1)]	41.5 ± 3.7	12.03 ± 2.5	6.15 ± 0.2
13	21.1	594	**Q-R-3-R-7**				
14	21.3	624	**I-G-3-R-7**	16.7 ± 4.3	20 ± 2.3	2.5 ± 0.4	1.5 ± 0.1
15	22.5	610	**Q-R-G-3**	0.7 ± 0.3	0.6 ± 0.13	0.1 ± 0.0	n.d.
16	23.9	464	**Q-G-3**	1.5 ± 0.1	2.0 ± 0.4	0.3 ± 0.1	0.1 ± 0.0
17	24	578	**K-R-3-R-7**	3.8 ± 0.3	1.1 ± 0.1	0.6 ± 0.1	0.3 ± 0.1
18	27.4	448	**K-G-3**	1.7 ± 0.1	12.1 ± 1.6	0.2 ± 0.1	0.4 ± 0.1
19	28	478	**I-G-3**	1.3 ± 0.1	2.6 ± 0.6	0.1 ± 0.0	< 0.1
20	32.8	432	**K-R-3**	1.0 ± 0.2	1.3 ± 0.2	0.1 ± 0.0	< 0.1
non flavonoids							
1	11.5	448	Glucobrassicin	5.6 ± 0.8	6.9 ± 0.8	4.7 ± 1.2	6.0 ± 1
6	15.8	478	4-Methoxyglucobrassicin	5.3 ± 1.3	5.4 ± 1.1	2 ± 0.1	2.2 ± 0.9
11	20.3	478	Neoglucobrassicin	42.3 ± 6.4	26 ± 5.0	8.8 ± 1.2	4.0 ± 2

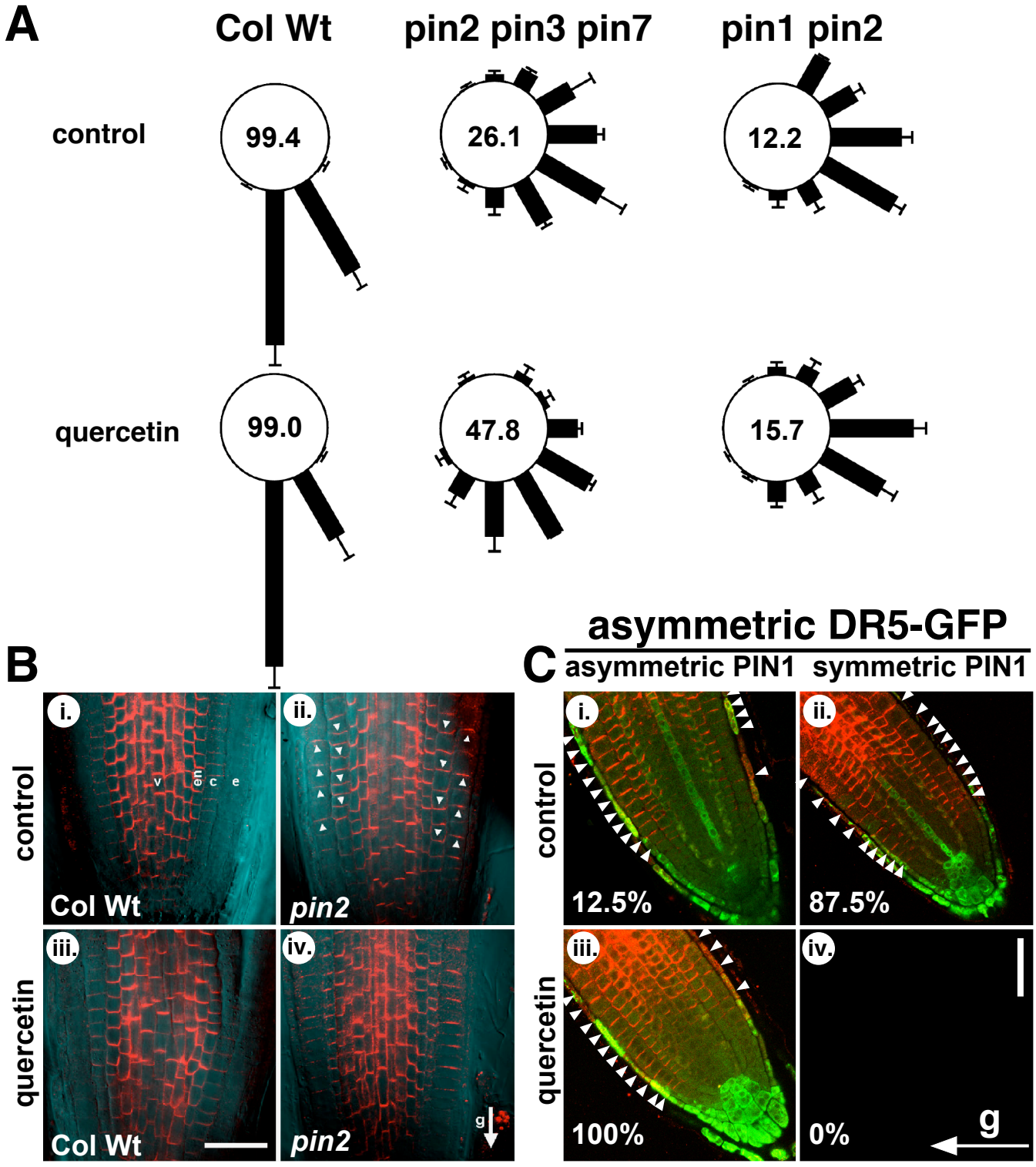
Table 1. Flavonoid derivatives detected in the MeOH extracts of entire root and RT-EZ of wild type and *pin2*.
The identification of the flavonoid derivatives was achieved by HPLC-UV(-)-MS/MS, comparison to reference compounds and/or according to (43-46). Each compound was quantified by integration of the corresponding signal area (x10^6 AU) presents in the

extract ion chromatogram (EIC, $[M-H]^-$) after HPLC-MS experiment. Values of entire roots represent means ± SE of at least three independent experiments (Student's *t*-test, $P<0.05$, $n=2$-4). Each extraction consists of a pool of 100 different roots. Quantification of RT-EZ flavonoid compounds is the result of at least two independent extractions in which each time 150 5mm-long root apices from 12 different agar plates were pooled. The natural flavonoid derivates described in this table have abbreviations as follows: G, glucose; K, kaempferol; I, isorhamnetin; Q, quercetin; R, rhamnose. Numbers indicate the position of glycosylation relative to the flavonol core. All flavonols identified are *O*-glycosylated.

[1)] Compound 12 are 13 are co-eluting in the HPLC-(–)-ESI-MS chromatogram and show the same quasi-molecular ions (*m/z* 593); therefore, they were integrated together.


A
EZ
RT
g
B
C
entire root
Total peak area
(mAU x 10^6 x 100 seedlings^-1)
RT-EZ
*
Col Wt
pin2
E
g
F
G
Total peak area
(rel. to vertical control)
*
Col Wt
pin2
D
Col Wt
Intensity x 10^6 [AU]
1
2
3
4
5,6
7
8
9
10
11
12
13
14
15
16,17
18
19
20
pin2
Intensity x 10^5 [AU]
Time (min)


A
Col Wt
pin2
Col Wt
pin2
control
99.4
33.5
0°
90°
quercetin
99.0
50.0
quercetin
+
NPA
28.6
13.0
kaempferol
95.5
52.1
NPA
39.4
14.7
Col Wt
pin2
B
QC
QI
col
pLRC
dLRC
control
quercetin
kaempferol
g


A
Col Wt
pin2 pin3 pin7
pin1 pin2
control
quercetin
99.4
26.1
12.2
99.0
47.8
15.7
B
C
asymmetric DR5-GFP
asymmetric PIN1
symmetric PIN1
control
quercetin
i.
ii.
iii.
iv.
Col Wt
pin2
12.5%
87.5%
100%
0%
g

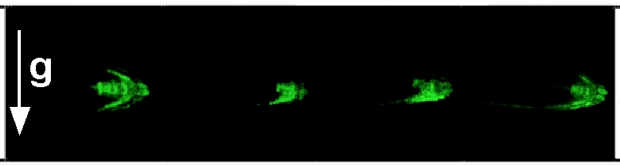
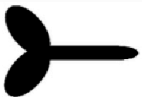
root tip orientation	0 1 2 3 				total (0+1+2+3)	% (2+3) of total (0+1+2+3)
Solvent control						
A 	3 17	10 3	17 1	2 0	32 21	59.4 4.8
B 	4 14	15 7	9 5	0 8	28 34	32.1 38.2
C 	0 4	0 2	0 4	0 0	0 10	0 40
Total	7 35	25 12	26 10	2 8	60 65	**46.7 27.7**
% of total	**11.7 53.8**	**41.7 18.5**	**43.3 15.4**	**3.3 12.3**		
Quercetin						
A 	12 11	27 9	32 12	6 4	77 36	49.6 44.4
B 	2 5	9 5	11 13	5 20	27 43	59.3 76.7
C 	1 5	0 3	0 7	0 6	1 21	0 61.9
total	15 21	36 17	43 32	11 30	105 100	**51.4 62**
% of total	**14.3 21**	**34.3 17**	**41 32**	**10.5 12.3**		

Table 2: The majority of quercetin-treated *pin2* roots form IAA gradients upon gravity stimulation.
Absolute and relative numbers of wild type (black) and *pin2* (red) seedling showing IAA gradients. Definition of IAA gradients is based on the strength of IAA movement from the LRC to the EZ, as assessed by confocal imaging of DR5-GFP fluorescent signals upon 2h gravity stimulation. Gradient intensity is quantified in arbitrary units (0= symmetric signal; 1= weak signal asymmetry, up to distal lateral root cap; 2= intermediate signal asymmetry, up to proximal lateral root cap; 3= strong signal asymmetry, up to elongation zone). Classification of root tip orientation is relative to the gravity stimulation vector, as depicted (A= neutral; B= towards the gravity stimulation vector; C= opposite to the gravity stimulation vector). Number of analysed wild type (*pin2*) seedlings was 60 (65) on solvent control and 105 (100) on 100 nM quercetin plates, respectively. Gravity vector (g) relative to the root is indicated by an arrow.

Flavonoids Redirect PIN-mediated Polar Auxin Fluxes during Root Gravitropic Responses

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Supplemental data

http://www.jbc.org/

Supplemental Figures S1, S2, S3

Supplemental Table S1

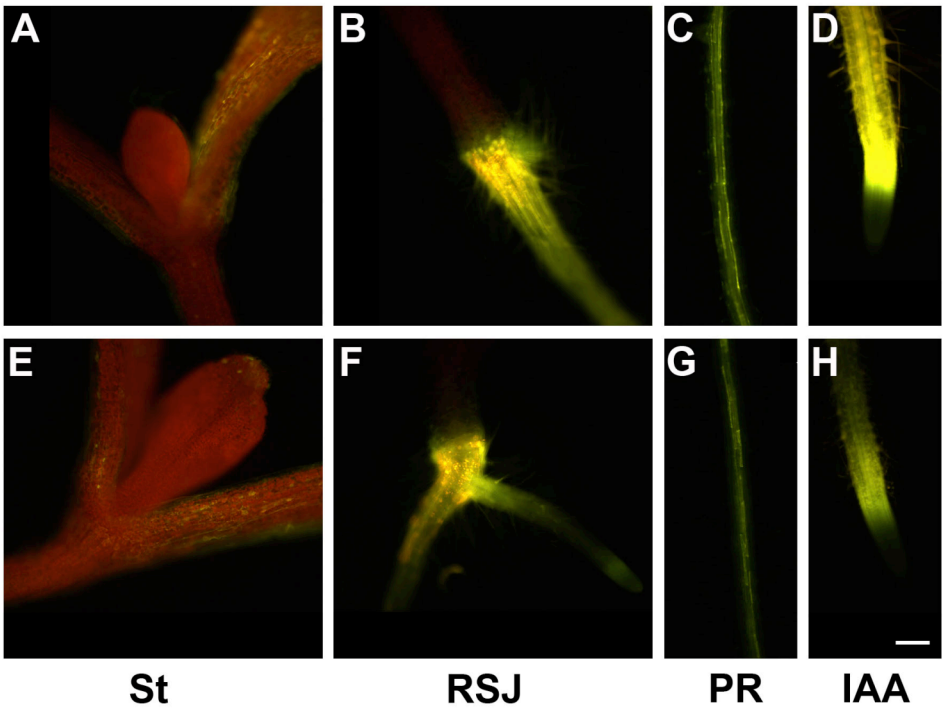


Figure S1. Flavonoid distribution in cotyledons, root-shoot junction and primary root of *pin2* mutant is not affected, but exogenous supply of IAA resulted in increased flavonoid accumulation in the EZ.
In situ flavonoid visualization of *Arabidopsis* seedlings using diphenylboric acid 2-aminoethyl ester (DPBA, a fluorescent dye that interacts with flavonoids) by epifluorescence microscopy (yellow fluorescence). These patterns were observed in all stained seedlings. **A-D**, wild type (Col Wt); **E-H**, *pin2*. St, stem; RSJ, root-shoot junction; PR, primary root; IAA, application of 100 nM IAA. Bar, 100 µm.

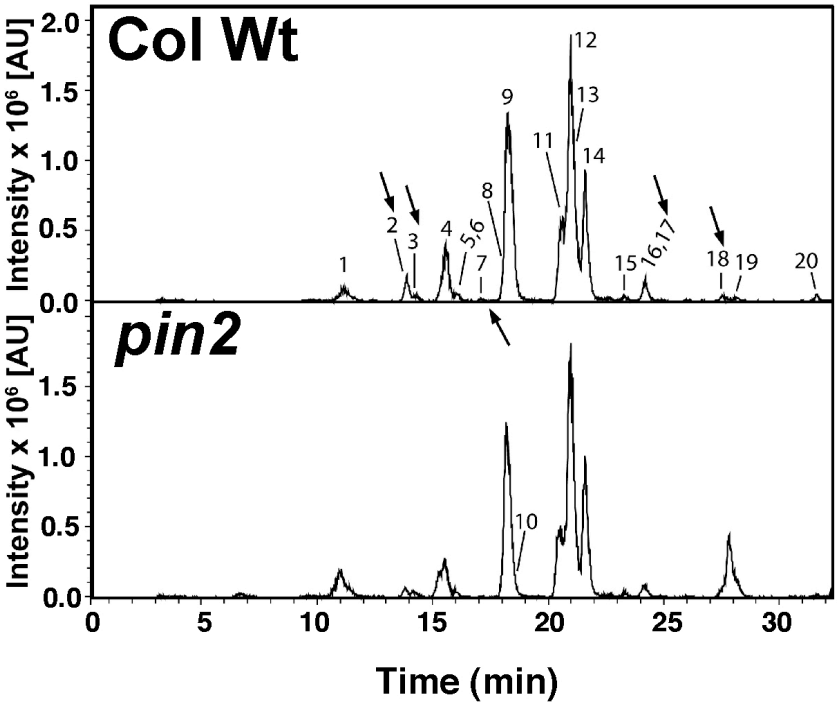


Figure S2. Accumulation of specific flavonoid glycosides in the entire *pin2* root is altered qualitatively but not quantitatively.
Representative sum of EIC of flavonoid derivatives found in wild type (Col) and *pin2* entire roots analysed by LC-ESI-MS. Selected ions are *m/z* 431, 447, 463, 477, 577, 593, 609, 623, 739, and 755, 771. Altered compounds are indicated by arrows. Peak numbers correspond to flavonoid derivates listed in Table 1. Note 10-fold higher intensities at the whole root level for *pin2* compared to the root tip-elongation zone (Fig. 1D).

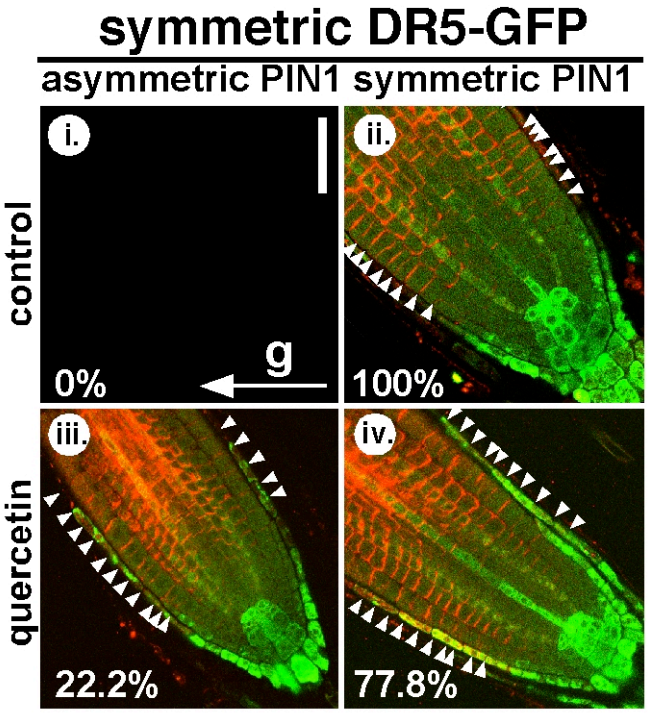


Figure S3. Flavonoid-dependent rescue of basipetal auxin reflux *pin2* is correlated with PIN1 asymmetric distribution across gravity-stimulated tissues.
Whole-mount *in situ* immunolocalization of PIN1 protein in *pin2* after 2h of gravity stimulation. 4 dag *pin2* seedlings were transferred on media supplemented with 100nM quercetin or the solvent (control) for 24h. Red, PIN1; green, $DR5_{rev}$-GFP expression. White arrows indicate more pronounced PIN1 proteins levels at the lower or upper side of gravity stimulated root tip. Bar, 30 mm. Gravity vectors relative to the root are indicated by an arrow. Percentages indicate relative occurrence of asymmetric or symmetric PIN1 distributions with symmetric DR5-GFP signals; the total number of analysed roots showing both clear DR5-GFP and PIN1 signals was 47.

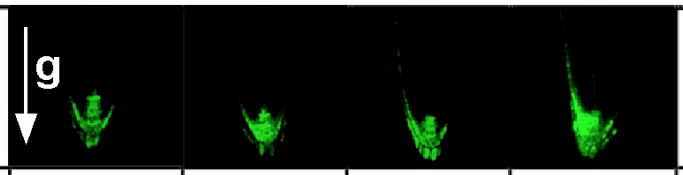
root tip orientation	0	1	2	3	total	% (2+3) of total
						
A 	51/ 12	5/ 3	0/ 4	0/ 0	56/ 19	0/ 21.1
B 	2/ 11	0/ 3	0/ 6	0/ 9	2/ 29	0/ 51.7
C 	1/ 10	0/ 4	1/ 0	0/ 1	2/ 15	50/ 6.7
total	**54/ 33**	**5/ 10**	**1/ 10**	**0/ 10**	**60/ 63**	**1.7/ 31.7**
% of total	**90/ 52.4**	**8.3/ 15.9**	**1.7/ 15.9**	**0/ 15.9**		

Table S1. Weak asymmetric IAA gradients were occasionally observed in vertically oriented *pin2* roots.
Absolute and relative numbers of wild type (black) and *pin2* (red) seedling showing IAA gradients. Definition of IAA gradients is based on the strength of IAA movement from the LRC to the EZ, as assessed by confocal imaging of DR5-GFP fluorescent signals upon gravity stimulation. Gradient intensity is quantified in arbitrary units (0= symmetric signal; 1= weak signal asymmetry, up to distal lateral root cap; 2= intermediate signal asymmetry, up to proximal lateral root cap; 3= strong signal asymmetry, up to EZ). Classification of root tip orientation is relative to the gravity stimulation vector, as depicted (A = neutral; B = to the left (lower than 90°); C = to the right (higher than 90°). Number of analysed wild type (*pin2*) seedlings was 60 (63). Gravity vector (g) relative to the root is indicated by an arrow.

3.7 The modified flavonol glycosylation profile in the Arabidopsis *rol1* mutants results in alterations in plant growth and cell shape formation

Christoph Ringli, Laurent Bigler, Ruth-Maria Leiber, Anouck Diet, **Diana Santelia**, Beat Frey, Stephan Pollmann and Marks Klein

THE PLANT CELL
AMERICAN SOCIETY OF PLANT BIOLOGISTS

In revision by the Plant Cell, resubmission encouraged

This paper offers a nice example on how flavonoids can play an important role as regulators of plant growth and development through auxin-induced and auxin-independent processes.

We showed that *rol1* mutants develop hyponastic cotyledons that are affected in pavement cell formation and thricome development. The observed phenotypes of *rol1* mutants correlate with an altered flavonol accumulation pattern. Blocking flavonoid biosynthesis suppresses the *rol1* shoot phenotypes, demonstrating that they are induced by the modified flavonol glycosides. Additionally, we found that auxin transport is inhibited in *rol1* mutants and that NPA-treated wild type seedlings developed hyponastic cotyledons, mimicking the *rol1* phenotype. Based on these results, we propose that the hyponastic growth may well be induced by the increased amount of auxin concentration, which is likely a direct result of the altered flavonol profile. In contrast, the aberrant pavement cell shape and thricome formation was not influenced by auxin, suggesting that flavonoids have additional functions in plant development control.

The modified flavonol glycosylation profile in the Arabidopsis *rol1* mutants results in alterations in plant growth and cell shape formation

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running title: Flavonols alter plant growth and cell shape

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ABSTRACT

Flavonoids are secondary metabolites known to modulate plant growth and development. A primary function of flavonols, a subgroup of flavonoids, is assumed to be the modification of auxin fluxes in the plant. In *Arabidopsis thaliana*, flavonols are glycosylated with glucose and rhamnose, and we show that the Arabidopsis rhamnose biosynthesis mutants *rol1* are affected in the glycosylation of flavonols. *rol1* mutants were initially isolated as suppressors of the root hair cell wall formation mutant *lrx1*. A more detailed analysis of the *rol1* mutants revealed hyponastic growth and aberrant pavement cell morphology in cotyledons and, in the stronger *rol1-2* allele, defective trichome formation. Blocking flavonoid biosynthesis suppresses the *rol1* shoot phenotypes, suggesting that they are induced by modified flavonol glycosides. Auxin concentration is increased in cotyledons of *rol1* mutants, indicating that flavonol species increased in *rol1* negatively regulate auxin transport. Experiments with the auxin transport inhibitor 1-*N*-naphthylphthalamic acid (NPA) reveal that the hyponastic growth but not the pavement cell or trichome formation phenotypes are induced by interfering with auxin transport. Thus, these results suggest that subtle changes in the composition of flavonol glycosides can have a tremendous impact on plant development through auxin-induced and auxin-independent processes.

INTRODUCTION

Flavonoids represent a highly diverse class of low molecular secondary metabolites of which >6000 different compounds have been described. They are important for pigmentation and UV-protection, serve as signals for pollinators and other beneficial organisms, participate in hormone signaling and function as phytoalexins. A number of biological processes such as transcriptional regulation, signal transduction, and cell-cell communication are influenced by flavonoids. Due to their antioxidant activity, an important role in plants appears to be the control of reactive oxygen species (ROS) (Lepiniec et al., 2006; Peer and Murphy, 2006). In *Arabidopsis thaliana*, 24 mutants were identified based on aberrant flavonoid accumulation (Routaboul et al., 2006). Frequently, they are defective in the biosynthesis of proanthocyanidins (Figure 1), the subgroup of flavonoids that holds responsible for the seed coloring (Korneef, 1990; Debeaujon et al., 2003; Lepiniec et al., 2006), and thus their seeds deviate from the typical brown color. The flavonols kaempferol, quercetin, and isorhamnetin constitute a subgroup of flavonoids that appears to be present in all tissues of Arabidopsis. They are *O*-glycosylated, mainly by glucose and rhamnose units at the C3 and C7 position, resulting in a large number of different molecules (Lepiniec et al., 2006). In Arabidopsis leaves, kaempferol glycosides, mainly kaempferol 3-*O*-glucoside-*O*-glucoside-7-*O*-rhamnoside (K-G-G-3-R-7), kaempferol 3-*O*-glucoside-7-*O*-rhamnoside (K-G-3-R-7), and kaempferol 3-*O*-rhamnoside-7-*O*-rhamnoside (K-R-3-R-7) are most abundant (Veit and Pauli, 1999; Jones et al., 2003).

Biochemical experiments and analyses of auxin fluxes in flavonoid deficient mutants suggest that flavonols negatively regulate auxin transport (Jacobs and Rubery, 1988; Brown et al., 2001; Buer and Muday, 2004; Peer et al., 2004). There is also evidence that flavonols directly influence cell growth. In petunia and maize, *chalcone synthase* mutants are blocked in the first step of flavonoid-biosynthesis (Figure 1) and are defective in pollen tube growth. While these plants are self-sterile, their pollen can partially grow on wild-type stigmas. Kaempferol was identified as the pollen germination-inducing constituent when applied to mutant stigma or added to the growth medium in *in vitro* pollen germination experiments (Mo et al., 1992). In addition, the petunia mutant is defective in root hair development, forming fewer and shorter root hairs compared to wild-type plants (Taylor and Grotewold, 2005).

Cell growth is largely determined by the extension rate of the cell wall, which is a complex structure composed of the polysaccharides cellulose, hemicellulose, and pectin, and a number of structural proteins (Carpita and Gibeau, 1993; Cassab, 1998). Several different components such as glycosylphosphatidylinositol (GPI)-anchored membrane proteins, wall associated kinases, and the microtubule and actin cytoskeletons have been shown to be important for cell shape determination (Wagner and Kohorn, 2001; Ringli et al., 2002; Mathur and Hülskamp, 2002; Smith, 2003). Recent data point towards an important role of ROS in the regulation of cell growth (Foreman et al., 2003; Gilmor et al., 2005; Roudier et al., 2005; Gapper and Dolan, 2006), suggesting that flavonoids might influence cell growth through modulating ROS levels (Peer and Murphy, 2006).

A model system to study the process of cell shape formation is the development of epidermal leaf pavement cells (Mathur, 2004). In Arabidopsis, pavement cells have a jigsaw puzzle-like shape where lobes extend into neighboring cells. This pattern is based on coordinated outgrowth at a given point in one cell with inhibition of outgrowth in the adjacent cell. The underlying signaling network involves a complex activation / inactivation interaction between ROP2 and RIC1 / RIC4 (Fu et al., 2005). ROP (Rho-related GTPase of plants) proteins control the organization of cortical microtubules and actin microfilaments, indicating that the cytoskeleton is a main determinant of cell growth (Smith, 2003; Fu et al., 2005). Indeed, interfering with actin filament nucleation changes pavement cell shape (Frank et al., 2003; Mathur et al., 2003; Djakovic et al., 2006). The well-known importance of the wall in cell-shape determination is reflected by the fact that the cellulose synthase mutant *rsw1* lacks lobe formation in pavement cells, resulting in straight cell boundaries (Williamson et al., 2001).

Previously, we have identified *rol1* mutants as suppressors of the Arabidopsis root hair mutant *lrx1* (Diet et al., 2006). *LRX1* encodes an extracellular protein specifically expressed in root hairs (Baumberger et al., 2001; Ringli, 2005). While *lrx1* mutants develop defective root hairs, *lrx1 rol1* double mutants show a suppressed *lrx1* phenotype and form wild type-like root hairs. Two *rol1* alleles were isolated and found to be mutated in *RHM1*, encoding one of three rhamnose synthase proteins in Arabidopsis that convert UDP-D-Glucose to UDP-L-Rhamnose (Diet et al., 2006; Oka et al., 2007). Rhamnose is an important component of pectin (Ridley et al., 2001) and the *rol1* mutants exhibit modifications in the pectin structure (Diet et al., 2006), which might be the molecular basis of the observed suppression of the *lrx1* root hair phenotype. Seedlings of both *rol1* alleles also develop cotyledons with an uneven surface and the peripheral zone bent

upwards. This is referred to as hyponastic growth and is the result of unequal growth of the adaxial and abaxial (upper and lower, respectively) side of the cotyledon surface (Kang, 1979). In addition, the stronger *rol1-2* allele develops slightly shorter roots and root hairs than wild-type seedlings (Diet et al., 2006).

In this work, we show that the *rol1* mutants are affected in cell shape control, which is reflected by oversized cotyledon pavement cells that lack the typical jigsaw puzzle-like cell shape. In addition, trichomes of the stronger *rol1-2* mutant are deformed. The fact that the main flavonols of the Arabidopsis shoot are rhamnosylated (Veit and Pauli, 1999; Jones et al., 2003) prompted us to determine the flavonol accumulation profile in the *rol1* mutants and investigate their influence on plant development. A targeted metabolon analysis revealed a modified flavonol-glycosylation pattern in *rol1* mutants. Genetic experiments suggest that the change in the flavonol profile induces the observed cotyledon and trichome phenotypes. Our results suggest that the shift in flavonols results in a change of auxin fluxes, which induces hyponastic growth in cotyledons. In addition, the modified flavonol profile appears to have a direct effect on cell formation that is independent of the effect on auxin, suggesting that flavonols can directly interfere with cell growth processes.

RESULTS

Flavonol accumulation is modified in *rol1* mutants

For the analysis of flavonol accumulation, wild-type, *rol1-1*, and *rol1-2* seedlings were grown for six days on MS-Agar plates in a vertical orientation. At this stage of seedling development, cotyledons are fully expanded and the first true leaves are about to form. The targeted metabolite profile analysis was performed by HPLC-MS as described in the METHODS section. Several changes in the profile could be observed between the wild type and the *rol1* mutants (Figure 2A). Some flavonols were less abundant while others were elevated in the *rol1* mutants compared to the wild type. Most flavonol glycosides could be identified according to their UV-absorption spectra, by MS-MS analysis, and by comparison to reference compounds of known chemical nature. The interpretation was also done according to recently published data (Kerhoas et al., 2006; Le Gall et al.,

2006; Stobiecki et al., 2006). The two *rol1* alleles showed a comparable profile, with differences only in the extent of the modification, i.e. the factor of increase or reduction in the amount of the flavonol species compared to the wild type. As shown in Figure 2B, a reduction of up to tenfold was found in *rol1* mutants for flavonols glycosylated with several rhamnose units. Interestingly, some flavonols containing single rhamnose units were increased in *rol1* mutants, such as K-R-3 by a factor of 10 and 4 for *rol1-1* and *rol1-2*, respectively. The most evident alteration in *rol1* mutants was found for the-3-*O*-glucosides of kaempferol, quercetin, and isorhamnetin, which are increased by a factor of around 40 compared to the wild type. To determine the tissues in which the different flavonols accumulate, seedlings were grown for six days in a vertical orientation, cut in the hypocotyl, and shoot and root tissue was analyzed separately. Of the 16 flavonol species detected in the extract, seven were present in comparable amounts in both samples, whereas K-R-3-R-7 and K-R-3-G-7 preferentially accumulated in the shoot and Q-G-R-R, Q-G-G-3-R-7, and I-G-3 preferentially in the root. Preferential accumulation was defined as fivefold or higher over-accumulation in one over the other tissue. Specific accumulation in only shoots or roots was found for only two compounds (K-R-3 and Q-R-G-3, respectively) (Figure 2B). Since the two *rol1* alleles showed comparable changes in the flavonoid profile, we decided to limit further analyses to the stronger *rol1-2* allele.

To investigate the distribution of flavonoids in vivo, wild-type and *rol1-2* seedlings were stained with diphenylboric acid-2-aminoethyl ester (DPBA), which forms fluorescent complexes with flavonoids. In agreement with previous reports (Peer et al., 2001), fluorescence in wild-type plants was found in the cotyledonary node, the root/shoot transition zone, and the root elongation zone. In *rol1-2* mutants, fluorescence was strongly increased in the root and observed in trichomes, which in wild-type plants are void of DPBA-detectable flavonoids (Supplemental Figure 1 online). No increase in DPBA-staining could be observed in the cotyledonary node.

rol1-2 mutants are affected in cotyledon pavement cell formation and trichome development

In addition to suppressing the *lrx1* root hair formation phenotype, *lrx1 rol1* double mutants show an aberrant cotyledon development, with an uneven cotyledon surface and hyponastic growth (Diet et al., 2006; Figure 3A and 3B). In addition, in *lrx1 rol1-2* but not *lrx1 rol1-1* seedlings, trichomes of the first rosette leaves are strongly deformed

(Figure 3D and 3E). The *lrx1* mutation present in the lines does not influence this or any other shoot phenotype, which is in agreement with the root-hair specific expression of *LRX1* (Baumberger et al., 2001).

To investigate whether these phenotypes are induced by the changes in the cell wall structure (Diet et al., 2006) or the modified flavonoid accumulation of the *rol1* mutants, the *lrx1 rol1-2* double mutant was crossed into the *tt4* mutant background. *tt4* plants carry a mutation in the *CHALCONE SYNTHASE* gene and are thus blocked in the first step of the flavonoid biosynthesis (Shirley et al., 1995; Figure 1). In *lrx1 rol1-2 tt4* triple mutants, flavonoid accumulation could no longer be detected by HPLC (data not shown) or by DPBA-staining (Supplemental Figure 1 online) and the hyponastic growth and trichome formation phenotypes observed in the *lrx1 rol1-2* mutants were fully suppressed (Figure 3C and 3F). This indicates that the *rol1-2* mutant phenotypes are related to the change in flavonol accumulation. To confirm co-segregation of the *tt4* mutation with suppression of the *rol1-2* mutant phenotypes, seedlings homozygous for *lrx1* and *rol1-2* but segregating for *tt4* were grown. Of 460 seedlings analyzed, 126 showed a lack of anthocyanin accumulation typical of homozygous *tt4* mutants (Shirley et al., 1995). Thus, the *tt4* mutant phenotype followed the 3:1 ratio (wild-type : mutant phenotype) expected for a recessive mutation. These 126 seedlings also developed wild type-like cotyledons and trichomes, indicating close linkage of the *tt4* mutation with suppression of the *rol1-2* mutant phenotypes.

To analyze the cotyledon growth phenotype in more detail, low temperature scanning electron microscopy (SEM) was used. At low magnification, an increase in cell size of adaxial cotyledon pavement cells was readily detectable in *rol1-2* mutants compared to the wild type (Figure 4A and 4B). At higher magnification, the jigsaw puzzle-like cell shape of wild-type pavement cells was apparent (Figure 4C). In *lrx1 rol1-2* plants, in contrast, pavement cell borders were straight and the characteristic lobes were absent (Figure 4D). In *lrx1 rol1-2 tt4* triple mutants, the pavement cells were reverted to wild-type shape (Figure 4F), again revealing suppression of the *rol1-2* mutant phenotype by *tt4*. No difference in pavement cell morphology was detectable between wild-type and *tt4* mutant plants (Figure 4C and 4E). This suggests that the absence of flavonoids in the *tt4* mutant does not influence cell formation and that the aberrant pavement cell shape is induced by the modified flavonol profile in *rol1-2* plants.

As an alternative, a rapid gel cast method (Horiguchi et al., 2006) was used to analyze pavement cell morphology, which confirmed the result of the SEM analysis.

Using this method, we confirmed that the shape of cotyledon pavement cells is indeed not influenced by *lrx1*, i.e. that *lrx1 rol1-2* and *rol1-2* plants show identical cotyledon phenotypes. Also, the same pavement cell phenotype as in *rol1-2* is found in *rol1-1* plants. The effect on pavement cell shape, however, is restricted to cotyledons. Rosette leaves formed by *rol1* seedlings do not develop aberrant pavement cells (data not shown).

The modified flavonoid profile of *rol1-2* does not influence root hair formation

Suppression of the *lrx1* root hair formation phenotype in the *lrx1 rol1* double mutants was initially attributed to modifications in the cell wall induced by the *rol1* mutations (Diet et al., 2006). The drastic effect on cell growth by the modified flavonoids in *rol1* mutants prompted us to investigate their role in root hair development and suppression of *lrx1*. As shown previously (Baumberger et al., 2001), *lrx1* mutants develop strongly deformed root hairs (Figure 5A and 5B). The *lrx1* root hair phenotype is suppressed in *lrx1 rol1-2* double mutants, where root hairs form again albeit with a reduced hair length (Diet et al., 2006; Figure 5C). The absence of flavonoids in the *lrx1 rol1-2 tt4* triple mutant does not influence suppression of *lrx1* through *rol1-2* (Figure 5F). Furthermore, the absence of flavonoids in the *tt4* single mutant does neither influence root hair formation (Figure 5A and 5D), nor the development of the *lrx1* mutant root hair phenotype (Figure 5B and 5E). In addition to modify root hair development, the *rol1-2* mutation causes reduced root growth (Diet et al., 2006). However, also this effect is not overcome by the *tt4* mutation, as *lrx1 rol1-2 tt4* triple mutants show a root length comparable to *lrx1 rol1-2* double mutants (data not shown). Hence, none of the investigated aspects of root (hair) development seem to be affected by flavonoids.

Auxin levels are increased in *rol1-2* mutants

Flavonols are known to negatively regulate auxin transport (Buer and Muday, 2004; Peer et al., 2004). Hence, we wanted to know whether the modified flavonol accumulation in *rol1-2* mutants influences auxin distribution. To this end, the β-glucuronidase activity induced by a *DR5* promoter:*GUS* fusion construct was compared in wild-type and *rol1-2* mutant plants, since the *DR5* promoter is induced by auxin signaling (Ulmasov et al., 1997). In wild-type seedlings, *DR5*-driven GUS activity was found at low levels in roots,

hypocotyls and cotyledons. In the *rol1-2* mutant, GUS activity was increased, particularly in cotyledons (Figure 6A). The identical experiment with the *rol1-1* mutant gave the same result (data not shown). Thus, auxin signaling is increased in *rol1* mutants, possibly reflecting increased auxin levels. To investigate this possibility further, we measured free auxin concentrations in cotyledons and roots of six-day-old seedlings. As shown in Figure 6B, the auxin concentration is indeed increased in cotyledons of *lrx1 rol1-2* mutants compared to either the wild type or *lrx1*. In roots, we did not detect a significant change in auxin concentrations. This led us to hypothesize that the modified flavonol accumulation in the *rol1-2* mutant results in reduced auxin transport and that the resulting increased auxin level causes the development of the *rol1-2* mutant phenotype. This view is supported by the reduced auxin concentration in the flavonoid-deficient *lrx1 rol1-2 tt4* triple mutant, which does no longer develop the *rol1-2* cotyledon and trichome phenotypes (Figure 6B).

In a next step, we wanted to test whether the effect of the *rol1-2* mutation on auxin transport and plant development can be mimicked by the auxin transport inhibitor 1-*N*-naphthylphthalamic acid (NPA). Wild-type plants containing the *DR5* promoter:*GUS* construct were grown for six days in the presence of 5 μM NPA. GUS activity was strongly increased in seedlings grown in the presence of NPA compared to control plants. Furthermore, NPA-treated seedlings developed hyponastic cotyledons (Figure 6C). In this respect, NPA treatment was able to phenocopy the effect of the *rol1-2* mutation. The pavement cell shape, however, was identical between NPA-treated and control seedlings, suggesting that the reduced auxin transport does not affect pavement cell morphology (data not shown). The same was found for trichome formation, which was not influenced by NPA (Supplemental Figure 2A and 2B online). To investigate whether the pavement cell and trichome formation phenotypes of the *rol1-2* mutant are caused by the combination of the effect of the *rol1-2* mutation on the cell wall structure (Diet et al., 2006) and auxin concentration (this work), *lrx1 rol1-2 tt4* triple mutants were grown in the presence of 5 μM NPA. NPA treatment induced hyponastic growth on cotyledons of the triple mutants, but it did neither affect pavement cell shape nor trichome development, which remained as in untreated triple mutant or wild-type seedlings (Supplemental Figure 2C and 2D online and data not shown). From these results, we conclude that hyponastic growth of cotyledons is induced by the increased auxin concentration, which is likely to be the result of the altered flavonols present in the *rol1-2* mutant. In contrast, the aberrant pavement cell shape and trichome formation

phenotypes appear to be auxin-independent effects of the flavonols present in the *rol1-2* mutant.

DISCUSSION

The Arabidopsis *rol1* mutants are affected in the *RHM1* gene which encodes a rhamnose synthase of Arabidopsis (Reiter and Vanzin, 2001; Oka et al., 2007). In addition to changing the structure of pectin (Diet et al., 2006), we show here that the *rol1* mutations result in a modified flavonol glycosylation profile. Thus, both groups of compounds known to contain considerable amounts of rhamnose are affected by mutations in *RHM1*. Flavonols glycosylated with multiple rhamnose units show the strongest reduction in *rol1* mutants, while those with single rhamnose units are much less affected and sometimes are even more abundant compared to the wild type. It can therefore be speculated that at limiting availability of UDP-rhamnose, production of the mono-rhamnosylated flavonols is still largely functional while the synthesis of di-rhamnosylated species is reduced. The concomitant increase in mono-glucosylated flavonols in *rol1* seedlings might be a compensatory effect of the limited rhamnose availability. The metabolite analysis suggests that flavonol glycosylation is altered with preferential conjugation at the C3-position and a redirection towards glucosylation. An accumulation of aglycon flavonols is not observed, demonstrating the metabolic plasticity in the glycosylation of flavonols. The possibility that the *rol1-2* phenotype is induced by a non-flavonoid compound that is modified as a secondary indirect effect of *rol1-2* can not be ruled out. However, the fact that the glycosylation pattern but not the overall presence of flavonols is affected in *rol1* mutants makes this scenario less plausible. Our assumption is further supported by the finding that the flavonoid-deficient *tt4* mutant does not induce abnormal cell formation but suppresses the effect of *rol1-2*. We propose that subtle changes in the flavonol conjugation pattern in *rol1*, i.e. the increase in one or several flavonol species, block normal cotyledon and trichome development, while complete removal of these conjugates allows the plant to resume normal development. It is an attractive hypothesis that under natural conditions, the plant has the potential to modify the flavonol glycosylation profile as a mean to modulate growth and development.

Auxin fluxes are affected in the *rol1* mutants

The *rol1* mutations cause an increase in auxin concentration in cotyledons, which induces hyponastic growth. The conclusion that this phenotype is caused by increased auxin levels is based on the finding that treatment of wild-type plants with the auxin transport inhibitor NPA can mimic this effect of the *rol1* mutations. Our data suggest that the flavonols accumulating in *rol1* reduce the flux of auxin. The *tt4* mutation, which blocks flavonoid biosynthesis (Shirley et al., 1995, Lepiniec et al., 2006), reverts the effect of *rol1-2* on auxin concentration and suppresses the *rol1* cotyledon phenotype. Indeed, auxin transport has been shown to be negatively regulated by flavonols and is increased in the *tt4* mutant compared to wild-type plants (Jacobs and Rubery, 1988; Brown et al., 2001; Buer and Muday, 2004; Peer et al., 2004). Recent experiments have shown that kaempferol and quercetin are able to compete with the auxin transport inhibitor NPA for a high affinity binding site in a protein complex containing AtPGP1, AtPGP2, and AtMDR1/AtPGP19. For AtPGP1 and AtMDR1/AtPGP9, a role in auxin transport has been demonstrated (Noh et al., 2001; Murphy et al., 2002; Geisler et al., 2005; Multani et al., 2003). Thus, flavonols are likely to directly modulate auxin transport, a process that is strongly influenced by the modified flavonol profile of the *rol1* mutants. They also influence localization of the auxin efflux facilitator protein PIN1 by modulating PIN1 vesicular cycling (Peer et al., 2004). Auxin is a phytohormone involved in a plethora of plant developmental processes and modifying auxin transport has a direct impact on plant growth (Friml, 2003). A hyponastic growth phenotype is also found in the Arabidopsis mutants *msg1* and *cnr1* that are both impaired in their response to auxin (Watahiki and Yamamoto, 1997; Laxmi et al., 2006).

Auxin-independent effects of flavonols on cell growth

In addition to the hyponastic growth phenotype, the flavonols in the *rol1* mutants also induce a pavement cell shape and trichome formation phenotype. Since treatment of wild-type plants with the auxin transport inhibitor NPA does change cotyledon growth but neither pavement cell nor trichome formation, we conclude that the latter effects are not caused by the reduced auxin transport. It is also unlikely to be the combination of increased auxin and the modification of the cell wall in *rol1* mutants (Diet et al., 2006) that causes the phenotypes, because treating the *lrx1 rol1-2 tt4* triple mutant with NPA induces the hyponastic growth but does neither affect pavement cells nor trichome

formation. Thus, the results suggest that the flavonols in the *rol1* mutants have a more direct effect on cell growth.

A small number of flavonol species are modified in the *rol1* mutants, suggesting that only few compounds might be required for the observed effect. Nevertheless, we are currently not able to identify the flavonol(s) that interfere with normal plant development, since glycosylated flavonols can not be exogenously supplied to the plant (Klein et al., 2000). Also, the mechanism by which flavonols modify cell growth remains to be elucidated. Flavonoids in general contribute to a reduction in reactive oxygen species, which have been shown to play an important role in cell growth (Foreman et al., 2003). Thus, the modification of the flavonol glycosylation pattern might influence redox-sensitive processes and thereby induce aberrant cell growth (Peer and Murphy, 2006). A number of biological functions such as transcription, translation, signal transduction, and vesicle trafficking are modified by flavonoids (Peer et al., 2004; Peer and Murphy, 2006). The latter process is particularly important, as cell wall material is transported by vesicles to the site of cell growth (Martin et al., 2001). This could explain the effect of flavonoid deficiency on pollen tubes and root hairs (Mo et al., 1992; Taylor and Grotewold, 2005), two cell types that expand rapidly by tip-growth (Yang, 1998) and therefore might be particularly sensitive to disturbed vesicle transport.

Most mutants affected in the pavement cell shape are impaired in the cytoskeleton. The *brick* and *arp2/3* mutants of maize and Arabidopsis fail to properly nucleate F-actin filaments (Frank et al., 2003; Mathur et al., 2003; Djakovic et al., 2006). The work by Fu et al. (2005) showed that both actin microfilaments and cortical microtubules are required for the formation of lobes and indentations, resulting in the jigsaw puzzle-like shapes typical for pavement cells. The actin cytoskeleton is well known for its vesicle-directing function during cell expansion (Staiger, 2000), indicating that vesicle trafficking might be affected in the pavement cell formation mutants. Thus, the same process, i.e. vesicle-transport and -cycling, might be affected in the *brick*, *arp*, and *rol1* mutants, even though the underlying reasons for the impairment are different. It is worth noting that the *arp2/3* and *rol1-2* mutants also share the aberrant formation of trichomes (Mathur et al., 2003 and this work), providing further evidence for a similarity between the processes affected in these mutants.

Root and shoot development of *rol1* mutants are affected in separate ways

The *rol1* mutants were initially isolated as suppressors of the root hair cell wall formation mutant *lrx1* (Baumberger et al., 2001; Diet et al., 2006). We assume that it is for the modified pectin structure caused by the *rol1* mutations that *lrx1* is suppressed in *lrx1 rol1* double mutants. The data presented here support this hypothesis since the flavonoid-deficient *lrx1 rol1-2 tt4* triple mutant still shows a suppressed *lrx1* phenotype. The reduced root growth observed for the *rol1-2* allele (Diet et al., 2006) is also maintained in the triple mutant, indicating that root and root hair development is not influenced to a detectable extent by the modified flavonol accumulation. Thus, the effect of flavonols on growth and development of Arabidopsis seedlings appears to be limited to the shoot.

The observed mutant phenotypes are specific for *rol1*

A number of flavonoid accumulation mutants have been described, many of which have mutations in genes encoding enzymes of the flavonoid biosynthesis pathway. The *tt* (*transparent testa*) mutants were identified based on alterations of the typical brown color of Arabidopsis seeds, which is caused by the polymerization of proanthocyanidins in the seed coat (Lepiniec et al., 2006). There are no reports on *rol1*-like phenotypes for *tt* mutants, suggesting that the aberrant development of *rol1* mutants is induced by the modified flavonol glycosylation profile. It is also possible that the combination of aberrant cell walls (Diet et al., 2006) and flavonol profile is required for the development of the *rol1* mutant phenotypes.

The results presented here demonstrate that flavonols can have an impact on auxin fluxes and cell developmental processes and that these two effects are partially independent. It will be interesting to characterize this finding in more detail as to which flavonol species induce(s) the *rol1* phenotypes and which molecular mechanisms trigger the aberrant development. To this end, we will try to manipulate the flavonol and flavonoid content by combining *rol1* with flavonoid-biosynthesis and regulatory mutants. The suppression of the *rol1* shoot phenotypes by restricting flavonol biosynthesis would allow narrowing down the number of candidate compounds for a role in interfering with plant development.

METHODS

Plant material and growth conditions

The *lrx1*, *rol1*, and *tt4* (*2YY6* allele) mutant lines used in this study are described elsewhere (Shirley et al., 1995; Baumberger et al., 2001; Diet et al., 2006). For the establishment of double and triple mutants, previously established molecular markers for *lrx1* (Diet et al., 2004) and *rol1-2* (Diet et al., 2006) were used to detect homozygous mutants. For *tt4*, the lack of anthocyanin staining in young seedlings and the yellow seed color were used as visual markers for selecting *tt4* homozygous mutants. For confirmation, we established a molecular marker for this *tt4* allele. A fragment encompassing the *tt4* mutation was amplified by PCR using the primers *tt4_F* and *tt4_R* (CCAACAGTGAACACATGACCGAC and GTTTCCGAATTGTCGACTTAGCGC, respectively). Two point mutations in the *tt4_R* primer compared to the *TT4* wild-type sequence created an *Eco* 47III restriction site in the PCR fragment produced from the mutant but not the wild-type genomic DNA.

For *DR5:GUS*, wild-type Columbia containing this construct (Ulmasov et al., 1997) was used for crossing with *rol1-2* and mutant lines homozygous for the transgene were subsequently selected. Identical T-DNA insertion patterns in the wild type and *rol1-2* were confirmed by southern blotting. For growth of plants in sterile conditions, seeds were surface sterilized with 1% sodium hypochlorite, 0.03% Triton X -100, stratified 3-4 days at 4°C, and grown for 5 days on half-strength MS-medium containing 0.6% Phytagel (Sigma), 2% sucrose, 100 mg/l myo-inositol with a 16 h light / 8 h dark cycle at 22°C. For crosses and propagation of the plants, seedlings were transferred to soil and grown in growth chambers with a 16 h light / 8 h dark cycle at 22°C.

Microscopy

Light microscopic observations were done with a Leica stereomicroscope MZ125. For observation of pavement cells, a gel cast was made of the leaf surfaces following an established protocol (Horiguchi et al., 2006), which was then observed by DICP microscopy using a Leica DMR microscope. Low temperature SEM was performed as described in Baumberger et al. (2001).

GUS- and DABP-staining

GUS staining was performed in 50 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 0.1% Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid between 2 h and 16 h at 37°C.

Flavonoid compound locations were visualized *in vivo* by the fluorescence of flavonoid-conjugated DPBA after excitation with blue light (Peer et al., 2001). Plants were grown for six days prior to staining. Fluorescent staining of whole seedlings was performed according to Buer and Muday (2004). Fluorescence was achieved by excitation with FITC filters (450 to 490 nm, suppression long pass 515 nm) on a Leica DMR fluorescence microscope and 10X or 20 X objectives. Digital images were captured with a Leica DC300 F charge coupled device (CCD) camera.

Flavonoid analysis and auxin measurement

For the analysis of the flavonol accumulation profile, seedlings were grown in a vertical orientation for six days on half-strength MS as described above. One hundred intact seedlings were pooled or seedlings were cut in the hypocotyl region and roots and shoots pooled separately in 500 µl 80% methanol. After an overnight incubation at 4°C, the plant material was macerated with a pestle, followed by vigorous vortexing. After pelleting the cell debris by centrifugation, the supernatant was transferred to a fresh tube and evaporated in a speed-vac centrifuge, with the temperature being limited to a maximum of 43°C. After evaporation, the pellet was resuspended in 100 µl fresh 80% methanol and used for analysis.

HPLC-MS analyses were performed on an Agilent 1100 HPLC system (Agilent Technologies) fitted with a HTS PAL autosampler (CTC Analytics), an Agilent 1100 binary pump, and an Agilent 1100 photodiode-array detector. Chromatographic conditions: Nucleosil 100-3 C18 column (3 µm, 2 x 250 mm, Macherey-Nagel); flow rate 0.170 mL min-1. Mobile phase: gradient within 25 min from 10 to 25% of solvent B, and then within 10 min from 25 to 70% of B (solvent A: 0.1% (v/v) HCOOH in H_2O), solvent B: 0.1% (v/v) HCOOH in MeCN). The HPLC was connected to a Bruker ESQUIRE-LC quadrupole ion trap instrument (Bruker Daltonik), equipped with a combined Hewlett-Packard Atmospheric Pressure Ion (API) source. The HPLC output was directly interfaced to the ESI ion source. The MS-conditions were: Nebulizer gas (N2) 40 psi, dry gas (N2) 9 l/min, dry temperature 300°C, HV capillary 4000 V, HV EndPlate offset -500

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V, capillary exit -100 V, skimmer1 -28.9 V, and trap drive 53.4. The MS acquisitions were performed in the negative electrospray ionization mode, at normal resolution (0.6 u at half peak height), under ion charge control (ICC) conditions (10'000) in the mass range from m/z 100 to 1000. The MS2 spectra were obtained in the auto-MS/MS mode. The isolation width was 4u, the fragmentation cut-off set by "fast calc", and the fragmentation amplitude set at 0.9V in the "SmartFrag" mode.

For auxin measurement, 250 mg root or hypocotyl/cotyledon tissue was collected, frozen in liquid nitrogen, macerated, suspended in 100% methanol, briefly warmed to 70°C, and kept for 30 min under continuous slight shaking. Prior to warming to 70°C, 100 pmol $[^2H]_2$-IAA was added as the internal standard. The GC-MS/MS analysis of IAA contents was carried out according to Müller et al. (2002). In brief, the samples were pre-cleaned by microscale solid-phase extraction on custom-made cartridges containing a silica-based aminopropyl matrix. After application of the samples and washing the microcolumn with 250 µL $CHCl_3$:2-propanol = 2:1 (v/v), the IAA fraction was eluted twice with 200 µL diethyl ether containing 2% acetic acid. Thereafter, the samples were taken to dryness, re-dissolved in 20 µL methanol, and treated with ethereal diazomethane. Subsequently samples were transferred to autosampler vials and excessive diazomethane and solvent was removed in a gentle stream of nitrogen. The methylated samples were then taken up in 10 µL of chloroform. Aliquots of 1 µL of each sample were injected into the GC-MS system for separation and mass fragment analysis using the autosampler and system quoted in the following. All spectra were recorded on a Varian Saturn 2000 ion-trap mass spectrometer connected to a Varian CP-3800 gas chromatograph equiped with a CombiPal autoinjector (Varian).

ACKNOWLEDGMENTS

We would like to thank Dr. Heller (GSF, Munich) and Dr. Veit (Kaufering) for providing us with flavonol standards that were invaluable for the flavonol structure analysis. We also thank Petra Düchting for technical support with the auxin quantification. This work was supported by the Swiss National Science Foundation grants No 31-61419.00 and 3100A0-103891 (C.R., R.-M.L., and A.D.) and 3100A0-116051 (M.K.).

FIGURE LEGENDS

Figure 1. Biosynthesis of Flavonoids in Arabidopsis.

Flavonoids are made of three molecules of Malonyl CoA and one molecule of *p*-Coumaroyl CoA. Flavonols are a subgroup of flavonoids and commonly consist of glycosylated kaempferol, quercetin, and isorhamnetin derivatives. *transparent testa* (*tt*) mutants of Arabidopsis are flavonoid biosynthesis mutants characterized by a change in seed color due to the absence of proanthocyanidins. The *tt4* mutant is deficient in chalcone synthase, resulting in the absence of flavonoids.

Figure 2. Flavonol Accumulation is Modified in *rol1* Mutants.

Flavonols were extracted from Arabidopsis seedlings grown for six days in a vertical orientation. **(A)** Flavonol elution profiles obtained from HPLC-(–)-ESI-MS analyses. In order to improve the selectivity, the sums of the extracted ion chromatograms corresponding to flavonoid derivatives 1 to 16 are displayed (EIC of *m/z* 431, 447, 463, 477, 577, 593, 609, 623, 739, 755, 771). Glucobrassicin derivatives [peaks Nr. 17 (*m/z* 447), 18, and 19 (both *m/z* 477)] are non-flavonoid compounds that are listed because they show the same mass spectra as some flavonoid derivatives. **(B)** The flavonol structure is given. The groups at the C7, C3, and C3' positions are listed in the table (X, Y, Z, respectively). The factor of induction / repression in *rol1* mutants compared to the wild type was determined on whole-seedling extracts. The substances are numbered according to the chromatograms in **(A)**. To determine tissue-specificity of flavonol accumulation, seedlings were cut in the hypocotyl and upper (shoot) and lower (root) tissue was collected and extracted separately. Detection in mainly one tissue was defined as a ≥5 fold overaccumulation of the compound in one tissue compared to the other. Only two compounds accumulated exclusively in one tissue (Nr. 11 and 13). K= kaempferol, Q=quercetin, I=isorhamnetin, G=Glucose, R=Rhamnose. * The exact structure of this compound could not be identified. ** The factor of induction / repression could not be determined because peak Nr. 7 and 9 are coeluting and have identical molecular weights.

Figure 3. Aberrant Cotyledon and Trichome Formation in *rol1-2* Mutants.

Cotyledons [**(A)** – **(C)**] and leaves [**(D)** – **(F)**] of five and eight day old Arabidopsis plants, respectively, reveal aberrant development in *rol1-2* mutants. In contrast to the wild type **(A)**, the periphery of cotyledons of *lrx1 rol1-2* mutants **(B)** bend upwards, referred to as hyponastic growth. Also the surface of *lrx1 rol1-2* mutant cotyledons is rough and contains bulging epidermal cells (arrows). This feature of *rol1-2* mutants is suppressed by the *tt4* mutation in *lrx1 rol1-2 tt4* triple mutants **(C)**. On the first leaves of *lrx1 rol1-2* mutants **(E)**, trichomes are deformed, in contrast to wild-type plants **(D)**. This phenotype of *rol1-2* is also suppressed in *lrx1 rol1-2 tt4* triple mutants **(F)**. Bar = 1mm.

Figure 4. Pavement Cell Phenotype in *rol1-2* Mutants.

Scanning electron microscopy (SEM) pictures from five-day-old cotyledons. Wild-type plants develop jigsaw puzzle-like pavement cells [**(A)** and **(C)**], whereas lobe formation is suppressed in *lrx1 rol1-2* mutants [**(B)** and **(D)**], resulting in brick-like cells. Also, cells are frequently oversized compared to the wild type. The pavement cell phenotype induced by the *rol1-2* mutation is suppressed by the *tt4* mutation **(F)**. *tt4* single mutants develop pavement cells that are indistinguishable from the wild type **(E)**. Bar = 250 μm [**(A)** and **(B)**] and 100 μm [**(C)** - **(F)**].

Figure 5. Flavonoid-Independence of Root Hair Development.

Seedlings were grown for five days in a vertical orientation. The *lrx1* mutant root hair phenotype [**(A)** and **(B)**] is suppressed by the *rol1-2* mutation **(C)**. This suppression is not dependent on the flavonoid-profile in *rol1-2* since the *lrx1* phenotype is also suppressed in *lrx1 rol1-2 tt4* triple mutants **(F)**. The absence of flavonoids in the *tt4* mutant does not have a significant effect on root hair formation **(D)**, nor does it influence the *lrx1* mutant phenotype **(E)**. Bar = 0.5 mm.

Figure 6. Auxin Levels are Modified in the *rol1-2* Mutant.

Seedlings transgenic for the *DR5:GUS* construct were grown for five days in a vertical orientation and stained for GUS activity. **(A)** A wild type is shown on the left, a *rol1-2* mutant on the right. **(B)** Auxin concentration in the different plant lines was measured for shoots and roots. The *rol1-2* mutation induces a significantly increased concentration of

auxin in the shoot. This effect is reverted by the presence of the *tt4* mutation. The *lrx1* mutation does not influence auxin concentrations in both tissues. **(C)** GUS activity is increased in wild-type plants containing the *DR5*:*GUS* construct when treated with 5 µM of the auxin transport-inhibitor NPA. NPA-treatment also induces the hyponastic growth phenotype in cotyledons as observed in *rol1-2* mutants **(A)**.

Supplemental Figure 1 online. Flavonoid Staining by DPBA in Arabidopsis Seedlings.

Seedlings of wild type, *lrx1 rol1-2* and *lrx1 rol1-2 tt4* mutants were stained with DPBA and analyzed by fluorescence microscopy. Roots of wild-type seedlings show moderate staining **(A)** while almost no fluorescence is observed in trichomes **(B)**. In *lrx1 rol1-2* mutants, fluorescence is strongly increased in roots **(C)** and in trichomes **(D)**. In the *lrx1 rol1-2 tt4* triple mutant, no fluorescence can be observed [**(E)** and **(F)**]. The root borders in **(A)**, **(C)**, and **(E)** are indicated with a fine line. Arrows in **(B)** and **(F)** point at trichomes. Bar [**(A)**, **(C)**, and **(E)**] = 0.5 mm, Bar [**(B)**, **(D)**, and **(F)**] = 0.5 mm.

Supplemental Figure 2 online. Effect of NPA on seedling development.

Wild-type and *lrx1 rol1-2 tt4* mutant seedlings were grown in the presence of 5 µM NPA in a horizontal orientation. Both the wild type and *lrx1 rol1-2 tt4* mutants show hyponastic growth but trichome formation is not affected. Bar = 1 mm.

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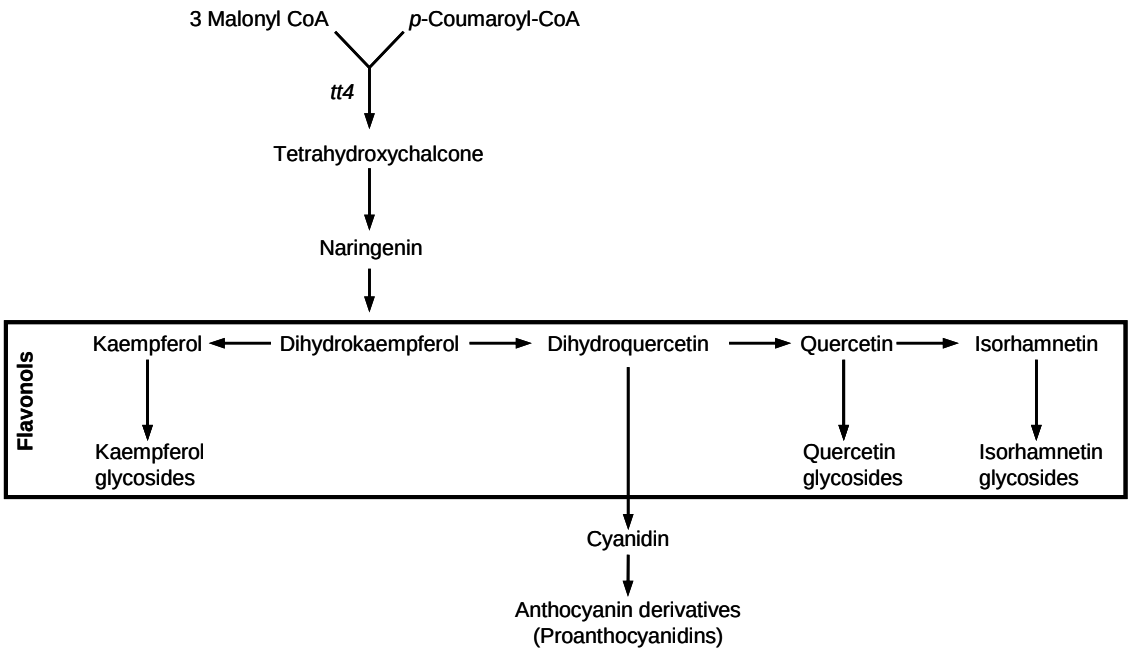
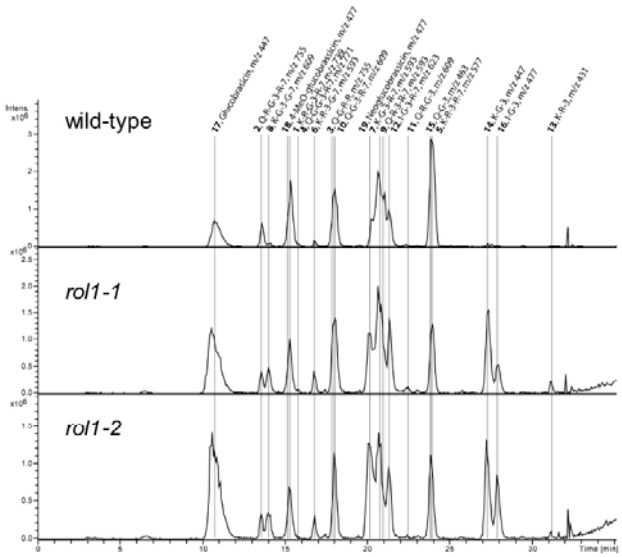
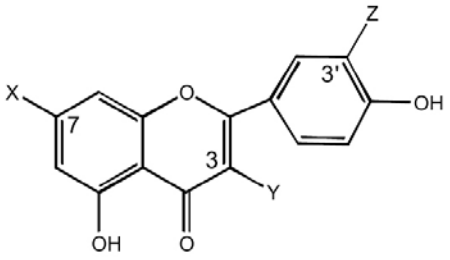

3 Malonyl CoA
p-Coumaroyl-CoA
tt4
Tetrahydroxychalcone
Naringenin
Flavonols
Kaempferol
Dihydrokaempferol
Dihydroquercetin
Quercetin
Isorhamnetin
Kaempferol glycosides
Quercetin glycosides
Isorhamnetin glycosides
Cyanidin
Anthocyanin derivatives (Proanthocyanidins)

Figure 1

A



B



Com-pound	MW	X	Y	Z	Trivial Name	factor of induction / repression in *rol1-1* seedlings compared to wild-type	factor of induction / repression in *rol1-2* seedlings compared to wild-type	tissue of detection in wild-type
1	740	α-rha	α-rha(1→2)β-glc	H	K-R-G-3-R-7	0.5	0.4	shoot and root
2	756	α-rha	α-rha(1→2)β-glc	OH	Q-R-G-3-R-7	0.6	0.6	shoot and root
3	756	?	?	OH	Q-G-R-R *	0.1	0.1	mainly root
4	772	α-rha	β-glc(1→6)β-glc	OH	Q-G-G-3-R-7	1.1	0.4	mainly root
5	578	α-rha	α-rha	H	K-R-3-R-7	0.2	0.1	mainly shoot
6	594	β-glc	α-rha	H	K-R-3-G-7	2.6	2.1	mainly shoot
7	594	α-rha	β-glc	H	K-G-3-R-7	**	**	
8	610	β-glc	β-glc	H	K-G-3-G-7	4.7	4.3	shoot and root
9	594	α-rha	α-rha	OH	Q-R-3-R-7	**	**	
10	610	α-rha	β-glc	OH	Q-G-3-R-7	0.9	0.6	shoot and root
11	610	OH	α-rha(1→2)β-glc	OH	Q-R-G-3	2.7	0.8	root
12	624	α-rha	β-glc	OCH3	I-G-3-R-7	1.6	1.2	shoot and root
13	432	OH	α-rha	H	K-R-3	10	4	shoot
14	448	OH	β-glc	H	K-G-3	40	40	shoot and root
15	464	OH	β-glc	OH	Q-G-3	40	50	shoot and root
16	478	OH	β-glc	OCH3	I-G-3	34	58	mainly root

Figure 2

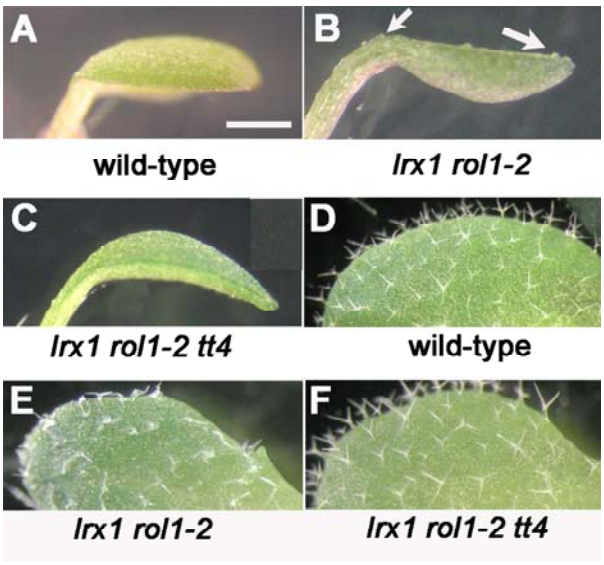


Figure 3

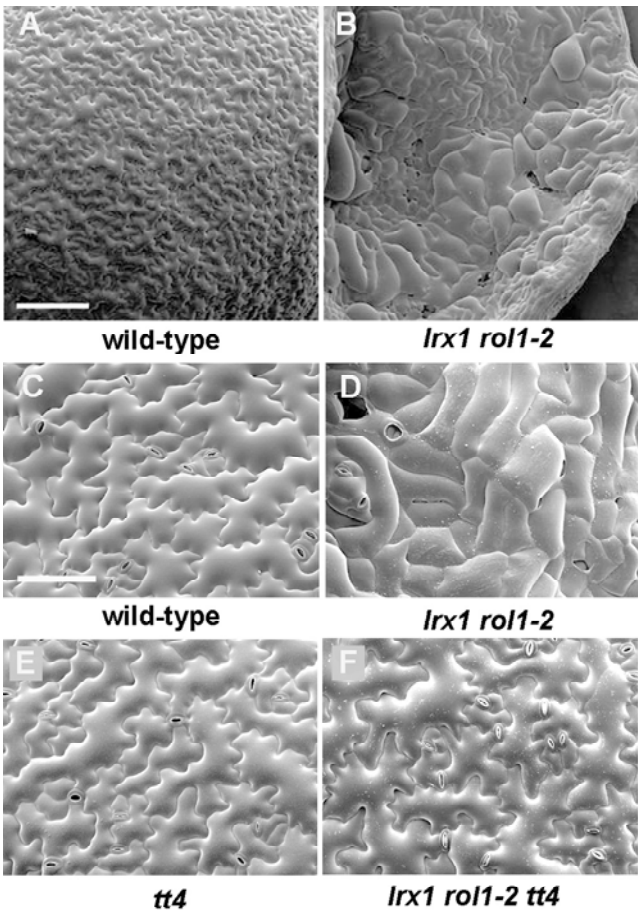


Figure 4

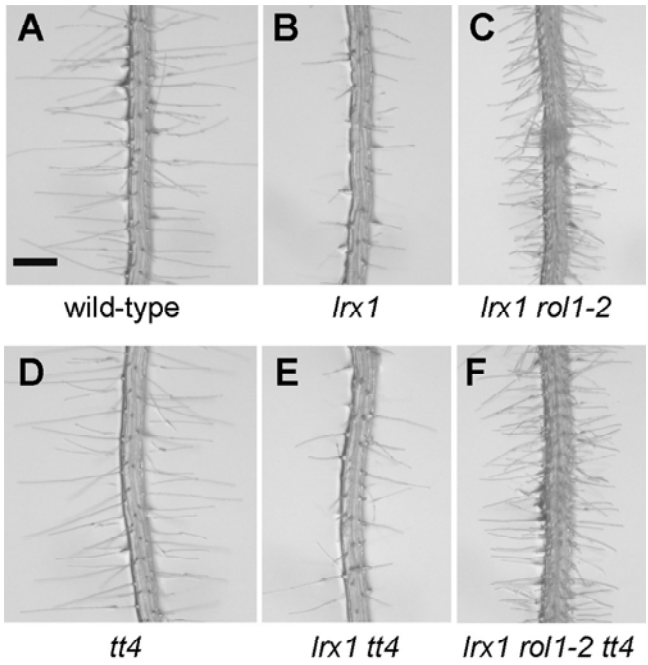


Figure 5

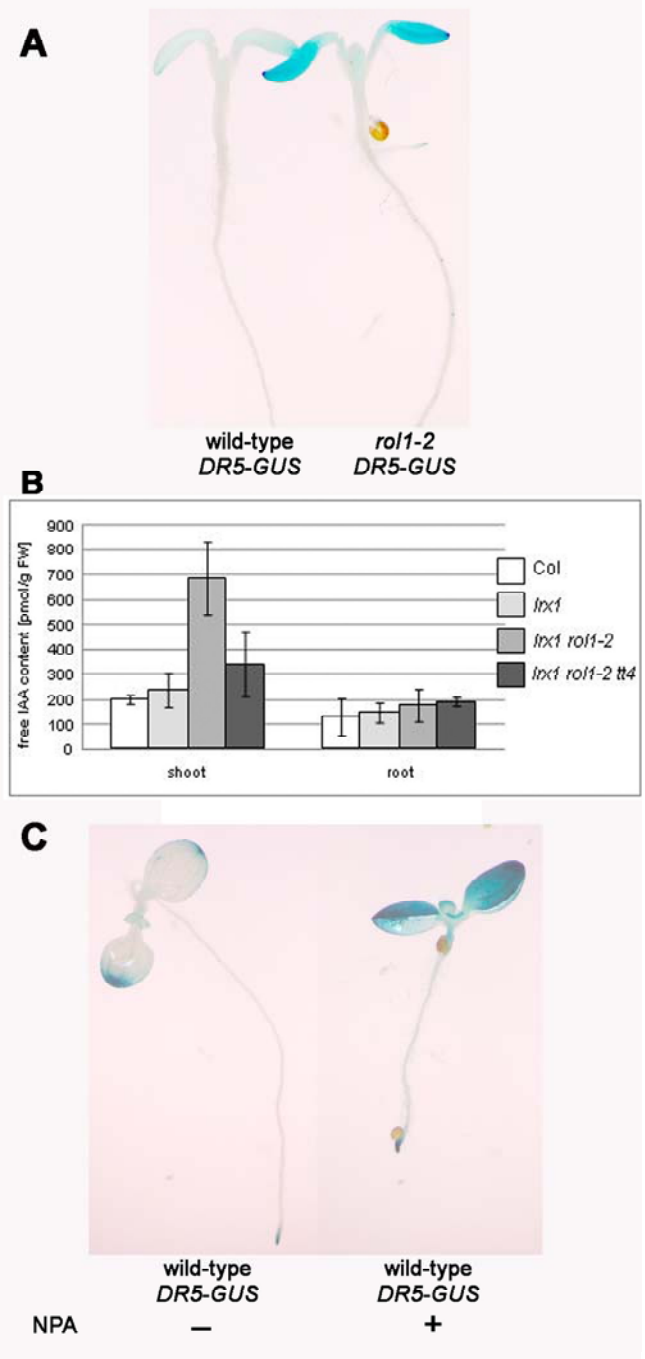
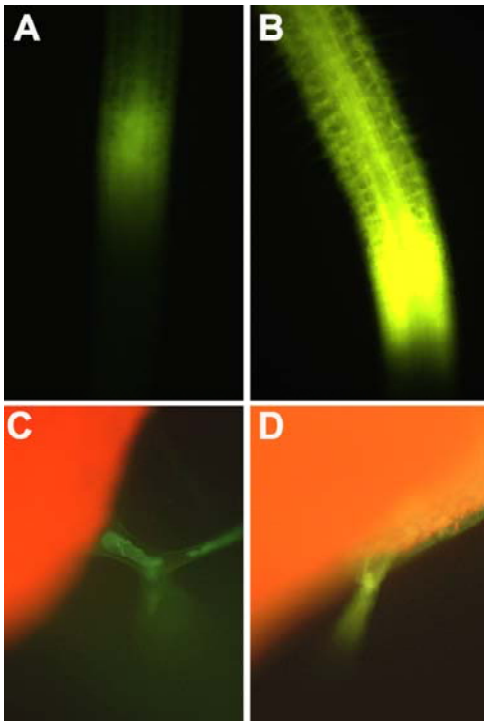
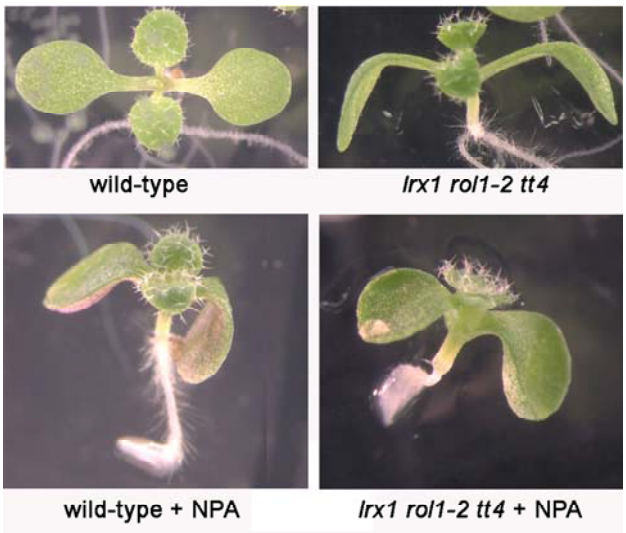

A
wild-type
DR5-GUS
rol1-2
DR5-GUS
B
free IAA content [pmol/g FW]
900
800
700
600
500
400
300
200
100
0
shoot
root
Col
lrx1
lrx1 rol1-2
lrx1 rol1-2 tt4
C
wild-type
DR5-GUS
wild-type
DR5-GUS
NPA
–
+

Figure 6



Supplementary data 1



Supplementary data 2

4 CONCLUSION AND OUTLOOK

Flavonoids are products derived from the secondary metabolism of plants, meaning that they are not fundamental to cell survival but are necessary components for survival in the environment in which plants grow. Invariably, plants are sessile organisms, unable to respond to evironmental changes via mobility, thus they have developed a plethora of cellular responses/mechanisms that enable them to react to environmental stimuli. In this respect, secondary metabolites such as flavonoids are an essential part of the adaptation mechanisms, exerting multifunctional roles as antipathogenicity, allelopathy and nodulation factors, regulators of plant growth and free radical scavenging. Consequently plants accumulate many different secondary compounds. Interestingly the diversity in these secondary metabolites is a result of simple differential modification of common precursors, with the resultant compounds having potentially divergent biological activitities. From an evolutionary perspective, it is clear that this strategy maximises the usage of secondary compounds with a minimal expenditure of energy.

During my PhD thesis, I devoted my efforts in the anaylsis of root flavonoids and their transport and role in intra and extra cellular signalling.

In the first period of my PhD work, in collaboration with two other PhD students in the laboratory, I developed a project about the characterization and the structural elucidation of the major isoflavonoids in white lupin cluster roots and their potential role in the rhizosphere as antifungal compounds.
Thanks to a fruitful collaboration with chemists from the University of Neuchâtel (Dr. E. Abou-Mansour and Prof. R. Tabacchi), we were able to identify by a LC-MS approach twelve major isoflavonoid compounds present in white lupin cluster roots and secreted into the rhizosphere upon growth under phosphorus deficient conditions. The compound dominating the HPLC profile, identified as genistein 6"-*O*-malonyl-O-glucoside, was found to be secreted in less abundance than genistein on a fresh weight basis (40 mg . g root FW . h^{-1}as compared to approx. 700 mg . g root FW . h^{-1}, respectively). Genistein 6"-*O*-malonyl-O-glucoside has a much higher UV absorbing capacity than genistein, but the latter was by far the isoflavonoid secreted in the highest amounts. In general, the amounts of secreted isoflavonoids by white lupin were significantly increased upon P deficiency, as previously reported in two other leguminous species, soybean (Murali and Teramura, 1985) and *Phaseolus vulgaris* (Juszczuk et al., 2004).

Secretion was significantly higher in cluster roots than in non cluster roots, especially for genistein, in agreement with previously published data (Neumann et al., 2000). However, the most interesting result of this study was the pattern of secretion along growing cluster roots: to date, studies have focused mainly on organic acid secretion and it was believed that most of the secretion processes occured in mature cluster roots. We demonstrated that maximal secretion of isoflavonoids occured in young and immature cluster roots. White lupin cluster roots thus appear to exhibit partition secretory activities, with younger roots secreting isoflavonoids and mature roots secreting organic acids. These findings extended the knowledge of the physiology of cluster-root exudation beyond the organic acids, and provided the field with new evidence about white lupin cluster roots as complex structures with a dual secretion activity, which is finely tuned along the different developmental stages.

Although cluster roots represent a successful strategy for phosphate acquisition in soil where P availability is low, the efficiency of organic acids as exchanger anions for phosphate depends largely on their stability and persistence in the soil. Since abundance of secreted carboxylates is mainly influenced by the biodegradation activity of soil microorganisms, we reasoned that white lupin might have evolved strategies to limit microbial degradation of organic acids. Isoflavonoids are known to be involved in plant microbe interactions, not only in the attraction of symbiotically nitrogen-fixing bacteria, but also in defense mechanisms against bacterial and fungal pathogens. We therefore decided to investigate the effect of these compounds on microbial and fungal growth.
We performed *in vitro* tests to assess the antimicrobial activity of the isolated isoflavonoids but observed no inhibitory growth effects on bacteria and fungi. This lack of antimicrobial activity of white lupin isoflavonoids may be related to the absence of prenylated isoflavonoids, which are known to be the most active antimicrobial compounds, particularly against fungi (Ingham et al., 1983; Tahara et al., 1994). Interestingly, in some fungal strains, a stimulation of sporulation could be observed and *Fusarium* species seemed to be the most susceptible to white lupin's isoflavonoids. Fungal sporulation can be viewed as a stress response and with respect to the microbial degradation of secreted citrate, sporulation is certainly beneficial to the plant since spores represent a dormant stage in the life cycle of fungi. Stimulation of sporulation occurred on TLC plates corresponding to the Rf value of the isoflavonoid with the highest UV aborption, identified as malonyl genistein. Unfortunately, we were unable to observe the

same effect when fractions where further purified, suggesting that more than one compound, co-migrating with malonyl genistein, was responsible.

To our knowledge, this is the first report of a role for isoflavonoids in protection of microbial organic acid degradations in cluster roots of white lupin. However, the precise identity of the compounds active in this inhibition remain unlucidated. Further experimentation will be necessary to uncover the identity and chemical properties of the compounds co-migrating with malonyl genistein on the TLC plates. NMR spectroscopy is an extremely powerful analytical technique for the determination of flavonoid structures, allowing complete assignments of all proton and carbon signals in NMR spectra of flavonoids isolated in the low milligram range. Excellent compilations of NMR data on individual flavonoids have previously been presented (Kamel, 2003; Tahara et al., 1994). Therefore, based on public available databases, it will be possible to define the structure of each isolated compound. Subsequently, it will be of interest to determine their role in the rizosphere of white lupin. The potential antimicrobial activity of each compound will be tested, and depending on the obtained results, more articulated experiments would be designed. For example, it will be of interest to test the persistence and stability of the organic acids secreted by lupin cluster roots in natural soils containing different microbial populations. This will allow uncovering a possible link between the secretion of specific isoflavonoids and the inhibition of the bacterial and/or fungal pathogens present in the different soils.

While the importance of flavonoid excretion from the roots is evident, the nature of the transporters is still unclear. Therefore, during my investigations on the role of flavonoids as signaling molecules, we decided to address the question of how flavonoids and root exudates in general are secreted into the rhizosphere. We approached this task as follows;

(a) In collaboration with the group of Professor Vivanco from the Colorado State University, we investigated the role of some members of the ABC transporters family in the secretion of phytochemicals from *Arabidopsis* roots;
(b) At the same time, I started the characterization of two members of the MATE tranporters family in white lupin as possible candidates as phenolics and/or organic acids transporters.

Since root exudation of phytochemicals is assumed to occurr near the root tip and the root elongation zone, and among the transporters responsible for their excretion, ABC transporters are promising candidates (Loyola-Vargas et al., 2007; Sugiyama et al., 2007), we chose ABC transporters specifically expressed in the epidermis of these root regions for a further analysis. We compared by an HPLC-MS approach the root exudation profiles of seven ABC transporter mutants with those of the wild type, and found that three phytochemicals were missing in the root exudates of the various mutants. One of the three compounds was tentatively identified as 3-hydroxy-4(Z), 6(Z), 8(Z), 10(Z)-tetratonic acid, using a hybrid quadrupole time-of-flight tandem mass spectrometry (QTOF-MS/MS). However, additional proof through NMR analysis is needed to confirm the proposed structure. Interestingly, none of these three compounds were present in the root tissues of the mutants or the wild type, suggesting that they are secreted directly after biosynthesis. All tested mutants were deficient in either one or two compounds, and the same compound was sometimes missing in more than one mutant, indicating that more than one ABC transporter can be involved in the secretion of a given phytochemical or that a transporter can be involved in the secretion of more than one secondary compound. In order to identify other compounds (both primary and secondary metabolites) in the exudates, the root exudates of the wild type and ABC transporter mutants were analyzed by GS-MS. Widespread changes in root secretion of primary and secondary metabolites were found in the GC-MS profiles of the ABC transporter mutants. However, it is possible that not all these changes are necessarily directly due to the specific ABC transporter, which correlates with the fact that differences were found only in the ratios of the same type of compounds while no missing compounds were indentified. The lack of expression of the transporter could change the expression pattern of other transporting systems, leading to changes in the secretion of several unrelated compounds. Collectively, these findings provide direct evidence that ABC-type transporters are involved in the release of secondary compounds from the roots. However, in this study the structural properties of two of the three phytochemicals absent in the root exudates of the mutants could not be determined, and for the third one only a tentatively proposed structure was given. When I compared the HPLC-MS profiles of these three compounds with the data obtained during the structural analysis of the flavonoid derivatives found in the *Arabidopsis* root extracts, I realized that at least based on the MS data, none of them is a flavonoid. This observation suggests that transporters belonging to the ABC transporters family might be involved in

the secretion of secondary compounds different from flavonoids, and that rather other type of protein such as MATE transporters or other non-pump related mechanisms may account for the secretion of flavonoids. Only a further chemical characterization of the identified compounds will help answering this question. Additionally, the experimental testing of their transport by individual ABC transporters will provide further insight into the involvement of this family of transporters in root exudation processes. To this aim, the use of yeast as heterologus expression system will be helpful to investigate the individual ABC transporters substrate specificity. However, it has been shown that some ABC membrane proteins, like PGP19 (Noh et al., 2001), cannot be expressed in yeast. In these cases, Hela cells can represent a valid alternative. If a robust protocol for Arabidopsis root protoplasts preparation will be generated, ABC transporter mutants will be directly test for their transport activity as compared to the wild type.

The idea that MATE transporters could be involved in flavonoid transport comes from the recent identification of the *TRANSPARENT TESTA12* gene in *Arabidopsis* which encodes a MATE transporter and is proposed to be involved in vacuolar deposition of proanthocynidin precursors in testa cells (Debeaujon et al., 2001).
Based on this information, I started to investigate whether lupin MATE genes that are specifically upregulated in cluster roots under phosphate stress could be involved in flavonoid and/or citrate transport. The preliminary data we collected demonstrated that *LaMATE1* and *LaMATE2* are specifically induced upon phosphate starvation, with the highest accumulation of mRNA transcripts in the mature cluster roots, where solubilization, soil extraction and uptake of phosphate into the plant occur. The localization of the closest *Arabidopsis* homolog (AtDXT12) to LaMATE2 and LaMATE1 at the plasma membrane further supported the idea of MATE transporters as candidates for citrate and/or isoflavonoid transporters. However, due to technical problems encountered during the molecular work and because white lupin was reported until very recently not to be transformable (Uhde-Stone et al., 2005), the evidence available thus far allows only for speculation.
Unfortunately, this part of my PhD work didn't bring any clear contribution to the field, but can be considered as a promising starting point for the characterization of the transport functions of the two lupin MATE transporters, as reported in detail in section 3.3. Currently, two PhD students in the laboratory are involved in this project.

In the second part of my PhD work I investigated the role of flavonoids in plant development, especially in their regulation of polar auxin transport in plant cells, including the characterization of a new auxin transport protein, AtPGP4.

The years 2005 and 2006 have been break-through years in auxin research. In addition to studies aimed to characterize in detail the function of PIN proteins and redundancy in plant development, the biochemical evidence of AUX1 mediating IAA uptake (Yang et al., 2006) and the identification of the TIR1/AFB family of auxin receptors (Dharmasiri et al., 2005), the research done in our laboratory with heterologous expression studies and mutant analysis has clearly demonstrated that AtPGPs function as primary active, catalytic components of auxin transport complexes, leading to a re-examination of the chemiosmotic models of polar auxin transport (Geisler and Murphy, 2006).

I contributed to the understanding of PGPs and their role in auxin transport characterizing a new member of the PGP subfamily of *Arabidopsis thaliana*, AtPGP4. By the analysis of *AtPGP4* loss-of-function mutants, we were able to show that *AtPGP4* functions primarily in the regulation of early root development. *atpgp4* plants revealed enhanced lateral root initiation and root hair lengths and showed altered sensitivities towards auxin and the auxin transport inhibitor NPA. Additionally, we found elevated free auxin levels and reduced auxin transport capacities.
Surprisingly, and in contrast to PGP1, which has been characterized as an auxin exporter (Geisler et al., 2005), all our transport experiments suggested an uptake direction, pointing to a role for PGP4 as an ATP-dependent IAA influx carrier, as further supported by the data obtained in the heterologous expression studies. Convincingly, concomitant to our study, work done in the laboratory of Professor Yazaki (Terasaka et al., 2005) demonstrated that PGP4 exhibited apolar localization in the cells within the lateral root cap and polar apical localization in epidermal cells of the root elongation zone, and that *atpgp4* mutants showed reduced basipetal auxin transport. Altogether, these data are consistent with PGP4 function as an auxin influx transporter, required for the basipetal redirection of auxin in the root. To our knowledge, these findings demonstrated for the first time that an ABC transporter catalyzes the cellular import of auxin.
Recently, a study from the group of Professor Spalding (Wu et al., 2007) used *pgp19* and *pgp4* T-DNA insertion mutants to distinguish between the roles of acropetal and

basipetal auxin transport in the branching of the root system. In agreement with our data, they found no difference between wild type and *pgp4* in primary root growth. However, they report that the loss of PGP4 had no detectable effect on lateral root branching, independently of the sucrose concentration in the growth media, and conclude that basipetal auxin transport have no visible effect on root system development and architecture. Conversely, acropetal auxin transport seems to play a major role during root branching (Wu et al., 2007).

Even though a series of transport studies *in planta* and in heterologus expression systems have provided concrete evidence that PGP proteins are mediators of auxin transport (Geisler et al., 2005; Santelia et al., 2005; Terasaka et al., 2005), only little mechanistic understanding is available so far. Detailed studies on PGP4, PGP1 and PGP19, in terms of substrate recognition and specificity, transport mechanism and structure, will surely be a major topic in the laboratory in the next few years. Much intense research ahead also will lie in the regulation of PGPs auxin transport acitivity. For example, it will be of interest to identify any further auxillary proteins which may either directly regulate PGPs function or which may facilitate protein trafficking. The mechanism of these interactions will also require elucidation. The existence of a reliable heterologous expression system in our laboratory will permit expansion into the biochemical characterization of the PGP proteins, providing a kinetic description of transport. Understanding the kinetics of the auxin transporters will make a great contribution to attempt to mathematically model auxin transport and distribution, with concomitant insights into plant regulation.

Another critical question that emerges is whether PGPs, AUX1/LAX and PIN proteins function independently or cooperatively in auxin transport. Previous reports suggested that PGPs regulate the plasma membrane stability of PIN proteins and that there might be specific PIN-PGP interactions (Noh et al., 2003), but only very rencently the extensive and complicated functional interaction between PIN- and PGP-based transport has indeed been demonstrated (Blakeslee et al., 2007). In this breakthrough study, the authors have examined the spatial, biochemical and functional relationships between different members of the PIN and PGP families i*n planta* and in heterologous expression systems. They also provided some *in vitro* evidence. More specifically, they have shown that the expression patterns of PGP1, PGP19, PIN1 and PIN2 in roots are partially overplapping. Phenotypic analysis of double and triple mutant combinations between different PGPs and PINs suggested synergistic interaction both in the root and in the shoot. Coexpression of PIN and PGP transporters in yeast and Hela cells revealed

a functional interaction affecting substrate specificity, inhibitor sensitivity and transport direction. Moreover, when AUX1 was co-expressed with PGP4, an additive effect on net IAA influx was observed, suggesting that a positive interaction between these two proteins may occur. However, the authors demonstrated that generally AUX1-mediated auxin transport does not involve direct PIN or PGP interactions (Blakeslee et al., 2007). Determing the developmental role and the molecular basis of such interactions will surely represent an important focus of auxin research in the next years.

The characterization of others members of the PGP subfamily, perhaps of those belonging to other clades (Geisler and Murphy, 2006) than PGP1 and PGP4 is also called for. These studies will bring additional light on the role of PGPs as auxin transport proteins. On the other hand, a possibility exists that other ABC proteins than the PGPs might have an auxin-transporter function. Indeed, there is a suggestion that a plant peroxisomal ABC transporter, AtPxa1 is involved in transport of indoyl-3-butyric acid into the peroxisome, where it undergoes a *b*-oxidation reaction to IAA (Zolman et al., 2001). Moreover, a recent report identifies a homologue of the yeast pleiotropic drug-resistance subfamily of ABC proteins, namely AtPDR9 (*A. thaliana* pleiotropic drug resistance 9), as being responsible for 2,4-D resistance (Ito and Gray, 2006). PDR proteins have been speculated to be involved in the detoxification of xenobiotics, however, little experimental support of this hypothesis has been obtained to date. This study demonstrates that 2,4-D, but not indole-3-acetic acid transport is affected by mutations in *pdr9*, suggesting that the PDR9 transporter specifically effluxes 2,4-D out of plant cells without affecting endogenous auxin transport. When incubated with [^{3}H]-NPA, *pdr9* roots accumulated dramatically more label than wild type roots, indicating that NPA transport may also be affected by the *pdr9* mutation (Ito and Gray, 2006).

In the auxin research field, flavonoids have been recognized to play an important role as endogenous regulators of polar auxin transport (Brown et al., 2001; Murphy et al., 2000). In particular, during my studies I was fascinated by the concept of flavonoids as positive mediators of plant gravitropism (Buer and Muday, 2004). Therefore, I tried to address this question in more detail. I investigated the role of flavonoids in root gravitropism by comparing the gravitropic response of *pin2* with wild type plants.

By means of an HPLC-ESI-MS approach and DPBA fluorescences studies, we were able to demonstrate that *pin2* mutants have impaired flavonoid glycosides accumulation patterns in the root tip-elongation zone (RT-EZ). Surprisingly, we found that low

concentrations of exogenous flavonoids can partially rescue *pin2* agravitropic phenotypes, by triggering the formation of lateral auxin gradients in a PIN2-independent manner. These results led us to hypothesize that in the *pin2* mutant exogenous flavonoids might re-activate gravitropic responses by the modulation of other PIN proteins, which, in the absence of a functional PIN2 protein, may functionally substitute PIN2. Therefore, we tested the gravitropic response of the triple mutant *pin2pin3pin7*, but found similar results to *pin2* single mutant, suggesting that neither PIN3 nor PIN7 are molecular targets of flavonoids. Thus, other proteins, such as PIN1, might represent the molecular target of flavonoids. Remarkably, exogenous flavonoids could not complement *pin1pin2* agravitropic phenotype, demonstrating that PIN1 is essential for flavonoid-dependent rescue of *pin2* gravitropism. Additionally, we found that chemical complementation by flavonoids strictly correlated with an asymmetric distribution of the PIN1 protein across the gravity stimulated roots, with stronger PIN1-specific signals at the lower side of the root. These findings suggest that PIN1 is the efflux complex component that facilitates basipetal auxin fluxes for gravitropic responses in flavonoid-treated *pin2* roots.

Although our results strongly indicate that flavonoids can redirect polar auxin transport during root gravitropic response in *pin2* mutant by promoting asymmetric PIN1 shifts, sheding new light on the role of flavonoids as regulators of polar auxin transport during root gravitropism, it remains to be determined how this is accomplished in detail. It has been shown that IAA reduces PIN1 gene expression in the roots (Peer et al., 2004), and this could be the mechanism by which PIN1 expression is induced in *pin2* mutant, which presumably has lower levels of auxin in the cells where PIN2 is missing. Peer et al (2004) also showed that PIN1 gene expression is depressed in *tt4* mutant, suggesting a positive regulation of the expression of this gene by flavonoids. Yet, how flavonoids lead to asymmetric PIN1 expression remains unclear. Two possible mechanisms are proposed. The first possibility is that flavonoids may affect the vesicular trafficking of auxin transport proteins, such as PIN1, leading to a relocation of the protein to the lower side of the epidermal cells upon gravity stimulation, thus activating the basipetal auxin transport required for the root tip bending. Peer et al (2004) indeed showed that flavonoids could modulate vesicular transport in the cells where they accumulate, maybe by regulating the activity of the protein required for trafficking. However, further experiments are necessary to test whether flavonoids alter the activities of these proteins by binding them directly or by altering their phosphorylation states.

A second possibility is that flavonoids may activate auxin proteins by modulating their phosphorylation state. Flavonoids are routinely used as both phosphatases and kinases inhibitors (Holder et al., 2007; Russo et al., 2003), so kinases and phosphatases associated with auxin transport are probable molecular targets of flavonoids action.

Studies in animal cell lines support the idea that flavonoids act on multiple kinase and phosphatase activities (reviewed in Williams et al., 2004). Apigenin inhibits protein kinase C (PKC) and MAPK (Huang et al., 1996; Kuo and Yang, 1995). For example, quercetin is routinely used as an inhibitor of mammalian PIPK, phospholipase A2, phosphodiesterases and PKC, by binding to the catalytic domain (Graziani et al., 1982; Tammela et al., 2004). Finally, kaempferol, quercetin, and genistein inhibit the CDC25A tyrosine phosphatase, a cell cycle-specific protein that is dephosphorylated in M phase (Aligiannis et al., 2001). In plants, the serine/threonine kinase PINOID (Benjamins et al., 2001; Christensen et al., 2000) which regulates PIN1, PIN2 and PIN4 activity by regulating the polarity of their subcellular localization on the plasma membrane (Friml et al., 2004), is the most likely candidate for flavonoid action, followed by the PINOID-related WAG kinases (Santner and Watson, 2006). Very recently, the work done in the laboratory of Professor Friml provided additional support to this hypothesis (Michniewicz et al., 2007). By a combination of genetic analysis, localization and *in vitro* and *in vivo* phosphorylation studies, the authors demonstrated that PINOID and the PP2A phosphatase both partially colocalize with PINs and act antagonistically on the their phosphorylation state, hence mediating apical-basal polar targeting. Specifically, PID-dependent phosphorylation leads to preferentially apical PIN localization, whereas low phosphorylation levels result in basal PIN targeting (Michniewicz et al., 2007). However, a direct proof of this regulation in *pin2* background is still missing.

In the last few months of my PhD work I tried to address the possibility that flavonoids act as modulators of phosphorylation in *pin2* background by carrying out gravitropic assays in the presence of chelerytrine or cantharidin, a protein kinase and phosphatase inhibitor, respectively (Castro et al., 1999; Rashotte et al., 2001). Upon treatments with chelerytrine or cantharidin, wild type gravitropic response was only slightly affected. Intriguingly, I found that exogenous supply of chelerytrine restored *pin2* gravitropic root tip bending similarly to quercetin. Consistently, treatments with chelerytrine triggered the enhancement of asymmetric *DR5-GFP* signals in the EZ of *pin2* gravity stimulated roots. In contrast, treatments with the protein phosphatase inhibitor cantharidin had no effect on *pin2* root gravitropims, substantiating physiological evidence that in *pin2* roots protein

phosphorylation plays a critical role in the suppression of the mutant agravitropic phenotype. It is therefore tempting to conclude that flavonoid-dependent PIN1 redistribution in *pin2* roots upon gravity stimulation might be accomplished by a direct or indirect modulation of PIN1 phosphorylation state. This hypothesis is being currently investigated by another PhD student in the laboratory.

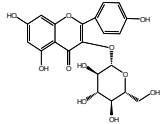
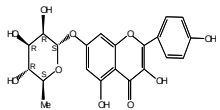
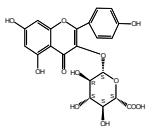
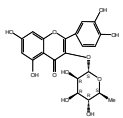
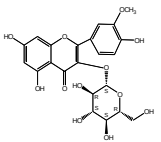
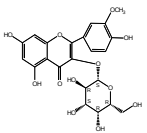
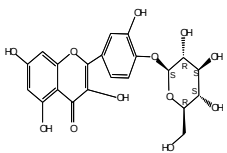
Along with the characterization of the role of flavonoids during root gravitropic responses, in collaboration with Dr. Christoph Ringli I investigated the possible contribution of flavonoids action on the regulation of cell growth and development. Previously in Christoph laboratory, a new gene – *LRX1* - involved in the regulation of the cell wall formation was identified and characterized (Baumberger et al., 2003a).
Few years later, in order to identify genes that are likely to be involved in the same developmental process as LRX1, a suppressor screen was carried out on the *lrx1* mutant and two independent *rol1* (for *repressor of lrx1*) alleles were identified (Diet et al., 2006). The *ROL1* locus encodes RHM1, which is involved in the formation of UDP-L-Rha. A more detailed analysis of the *rol1* mutants by Ringli and co-workers revealed hyponastic growth, aberrant pavement cell morphology in cotyledons and defective thricome formation. The fact that the main flavonols of the Arabidopsis shoot are rhamnosylated (Jones et al., 2003; Veit and Pauli, 1999) led to the hypothesis that an alteration in flavonoids accumulation in the *rol1* mutants might occur and have an influence on plant development. We performed a targeted metabolite profile analysis of *rol1* mutants by HPLC-ESI-MS/MS and found that *rol1* mutants accumulate five times less oligo-rhamnosylated flavonols and fourty time more glucosylated flavonols. This altered flavonol accumulation pattern of *rol1* mutants correlated with the developemt of hyponastic cotyledons that are affected in pavement cell formation and thricome shape. Blocking flavonoids biosynthesis by crossing the *rol1* mutant with the flavonol free *tt4* mutant suppressed the *rol1* shoot phenotypes, demonstrating that they are specifically induced by the modified flavonol glycosides. Additionally, we found that auxin transport was inhibited in *rol1* mutants and that NPA-treated wild type seedlings developed hyponastic cotyledons, mimicking the *rol1* phenotype. Based on these results, we concluded that the hyponastic growth might be induced by the increased amount of auxin concentration, which is likely a direct result of the altered flavonol profile. In contrast, the aberrant pavement cell shape and thricome formation was not influenced by auxin, suggesting that flavonoids have additional functions in plant development control.

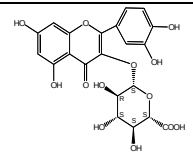
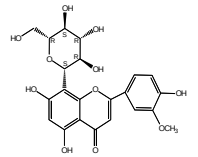
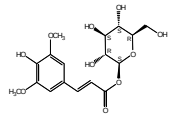
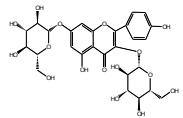
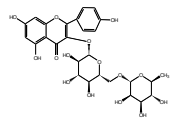
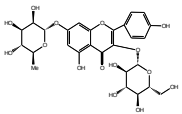
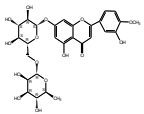
This study represents one of the few examples showing that flavonoids can have a simultaneous impact on auxin fluxes and cell developmental processes, and that these two effects are partially independent. In this respect, flavonoids can be considered as high versitale regulating molecules of plant growth and development. It is an attractive hypothesis that under natural conditions the plant has the potential to modify the flavonol glycosylation pattern as a mean to modulate growth and development.

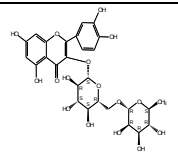
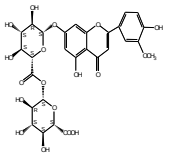
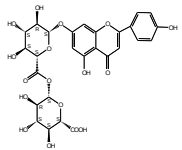
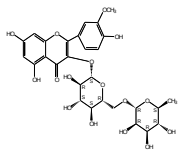
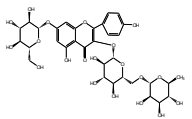
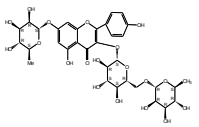
As a next step, it would be interesting to test which of the identified altered flavonol compounds induce the *rol1* phenotypes and which molecular mechanisms trigger the aberrant development. However, it is very often difficult to isolate enough amounts of pure compounds from plant crude extracts, and so far, not many glycosylated flavonol standards are commercially available. It will be also of interest to create multiple mutants combinations between *rol1* and different flavonoid deficient mutants, aiming to identify possible enhancers or suppressors of the observed phenotypes.

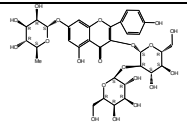
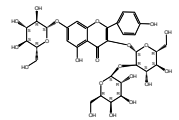
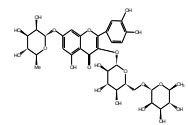
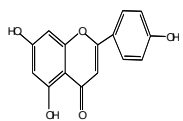
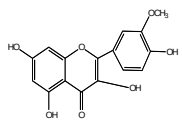
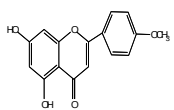
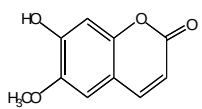
5 APPENDIX A

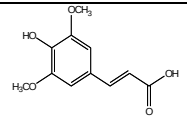
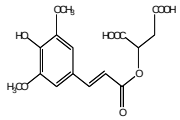
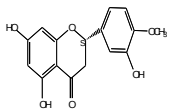
HPLC-ESI-MS/MS analysis of flavonoid standards used as reference compounds for Arabidopsis root flavonoids identification

Compound	Origin	CAS-Nr	Rt [min]	MW	(+)-ESI	(–)-ESI	(+)-MS/MS	(–)-MS/MS	UV-ABS [nm]	Structure proposal
Monoglycoside										
Kaempferol-3-glu + Na	Veit	480-10-4	27.3	448		447.0		327; 285; 255; 227; 171	320S; 288; 247min; 224S; 211; 205min; 195	
Kaempferol-7-rha	Diana – ref19	20196-89-8	32.8	432		431.1		285; 284; 257; 151	364; 331S; 287min; 267; 255S; 236min; 218S; 198	
Kaempferol-3-glucoronide	Diana – ref20	no CASnr	27.2	462		461.1		285; 257; 175	347; 305S; 276min; 265; 270min; 205S; 197	
Quercitrin or Quercetin-3-Rha	Extrasynth	522-12-3	27.4	448		447.2		343; 301; 271; 255; 179; 151	337; 300S; 282min; 268; 247min; 212; 203min; 196	
Isorhamnetin-3-glu	Extrasynth	5041-82-7	27.9	478		477.0		357; 329; 315; 314; 299; 298; 285; 271; 257; 243	353; 297S; 277min; 266S; 253; 227min; 204	
Cacticin or Isorhamnetin-3-gal	Diana – ref4 (not clean)	6743-92-6	27.1	478		477.0		357; 329; 315; 314; 300; 299; 285; 271; 243		
Quercetin-4'-Glu	Diana - ref5	20229-56-5	28.8	464		463		301; 179; 151	365; 315S; 283min; 262S; 254; 238min; 202	

Compound	*Origin*	*CAS-Nr*	*Rt [min]*	*MW*	*(+)-ESI*	*(–)-ESI*	*(+)-MS/MS*	*(–)-MS/MS*	*UV-ABS [nm]*	*Structure proposal*
Quercetin-3-glucoronide	Diana – ref18	no CASnr	23.7	478		477		301; 179; 151	355; 309S; 282min; 256; 268min; 203	
Scoparin or Chrysoeriol-8 C-glu	Diana – ref11	301-16-6	24.3	462		461		341; 326; 313; 298	347; 296S; 284min; 268; 263min; 250; 240min; 210	
1-Sinapoyl glu or sinapic acid 4-O-glu	Diana – ref24	14364-09-1 or 117405-52-4	14.1	386		385.1		325; 295; 265; 247; 232; 223; 205; 190; 164; 149	332; 267min; 240; 230S; 213; 197	
Diglycosides										
Kaempferol 3,7 diglu	Veit	25615-14-9	13.9	610		609		489; 447; 327; 285	347; 329S; 285min; 265; 247S; 237min; 220S; 198 (from Diana – ref17)	
Kaempferol 3-rha-glu / Kaempferol-3-rut	Veit / Diana – ref2	17650-84-9	25.8	594		593.1		285	320S; 288; 247min; 224S; 211; 205min; 195	
Kaempferol-3-Glu-7-Rha	Veit	2392-95-2	20.7	594		593.1		447; 431; 285	346; 320S;284min; 267; 248S; 238min; 221S; 198 (from Diana– ref 15)	
Diosmin	Guggi	520-27-4	28.0	608	609.1	607.1	463; 313; 301; 286; 258	341; 299; 284	–	

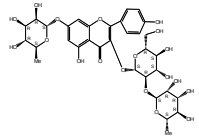
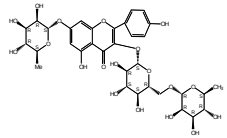
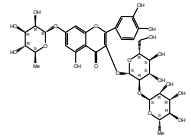
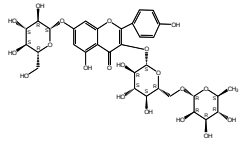
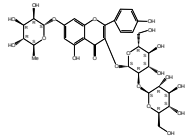
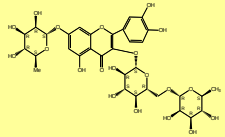
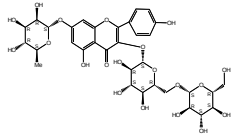
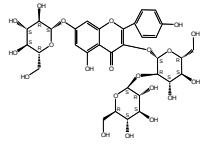
Compound	*Origin*	*CAS-Nr*	*Rt [min]*	*MW*	*(+)-ESI*	*(–)-ESI*	*(+)-MS/MS*	*(–)-MS/MS*	*UV-ABS [nm]*	*Structure proposal*
Rutin or Quercetin 3-rut (Rha-glu)	Diana – ref1	153-18-4	22.2	610		609.2		343; 301; 271; 255; 179	356; 307S; 280min; 256; 238min; 207 (also Diana-ref23)	
Chrysoeriol-7-diglucoronide???	Diana – ref7	no CAS nr!	22.3	652		651		633; 517; 395; 371; 351; 341; 285; 193	347; 295min; 266; 262min; 252; 237min; 207	
Apigenin-7-diglucoronide (described as Apigenin-7-glucoronide)	Diana – ref8	no CAS nr!	21.6	622		621.1		487; 351; 311; 289: 269; 193	338; 292min; 265; 247min; 198	
Narcissin or Isorhamnetin-3-rut (Rha-glu)	Diana – ref3	604-80-8	26.2	624		623.2		315; 300; 271; 255; 243	354; 311S; 277min; 264S; 254; 237min; 206	
Triglycosides										
Kaempferol-3-Rut-7-Glu (Rut=rha-glu)	Veit	34336-18-0	14.0	756		755.2		593; 285	347; 330S; 285min; 267; 248S; 238min; 220S; 198 (from Diana – ref16)	
Kaempferol-3-Rut-7-Rha	Diana – ref14	57526-56-4	20.5	740		739.3		593; 285	347; 332S; 285min; 267; 248S; 238min; 222S; 198	

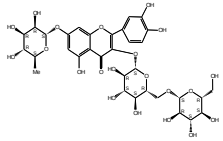
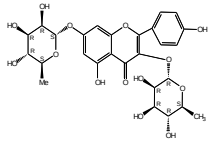
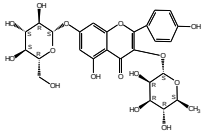
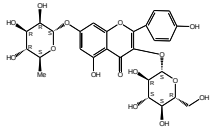
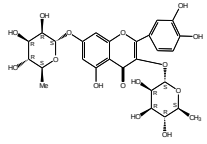
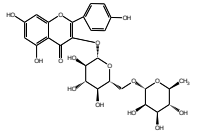
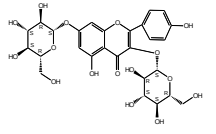
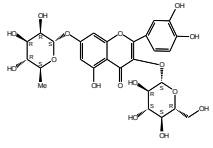
Compound	*Origin*	*CAS-Nr*	*Rt [min]*	*MW*	*(+)-ESI*	*(–)-ESI*	*(+)-MS/MS*	*(–)-MS/MS*	*UV-ABS [nm]*	*Structure proposal*
Kaempferol-3-sophoroside-7-rhamnoside (soph=2-O-3 O-diglu)	Diana – ref10	93098-79-4	14.3	756		755.3		609; 429; 285; 284	345; 324S; 287min; 365; 245S; 238min; 220S; 197	
Kaempferol-3-sophoroside-7-glucoside (soph=2-O-3-O-diglu)??	Diana – ref13	55136-76-0	8.5	772		771.2		609; 429; 327; 285; 284; 257; 255	346; 327S; 286min; 266; 247S; 238min; 222S; 197	
Quercetin-3-rutinoside-7-rhamnoside Kämpferol-3-Rut?-7-Rha bezeichnet die Probe	Veit	57528-70-8	17.9	756		755.3		609; 447; 301	354; 292S; 283min; 267S; 257; 228min; 205	
Aglycone										
Apigenine	Extrasynth	520-36-5	34.8	270	271.0	268.9	252; 153; 121	225; 201; 183; 151; 149	348; 312S; 282min; 251; 235min; 208; 203min; 198	
Isorhamnetin	Fluka	480-19-3	35.2	316		315.0		300	372; 335S; 311S; 284min; 263S; 254; 248; 220	
Acacetin	Guggi	480-44-4		284	285.0	282.9	270; 265; 247; 242; 153	267.8	331; 304S; 282min; 269; 332; 208; 199min; 195	
Scopoletin	Diana – ref27	92-61-5	22.4	192		191.0			345; 307min; 295; 270min; 253S; 228; 218min; 205	

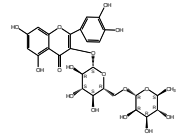
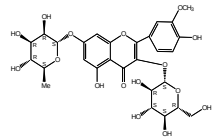
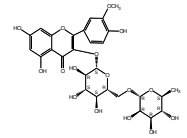
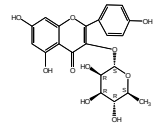
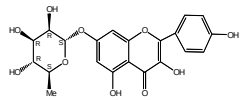
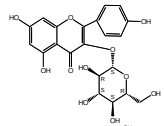
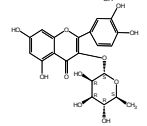
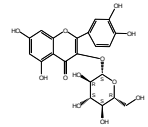
Compound	*Origin*	*CAS-Nr*	*Rt [min]*	*MW*	*(+)-ESI*	*(–)-ESI*	*(+)-MS/MS*	*(–)-MS/MS*	*UV-ABS [nm]*	*Structure proposal*
Cinnamic, Sinapic or Sinapinic acid	Diana – ref26	530-59-6	22.4	224		223.0; 208.0; 193.0			325; 263min; 239	
Sinapoyl malate	Diana – ref25	92344-58-6	24.5	340		339.0			330; 265min; 222	
Hesperetin	Guggi	520-33-2	35.2	302		301.0		286; 283; 268; 258; 257; 242; 233; 227; 215; 199; 174; 164; 125	323S; 288; 250min; 226S; 213; 207min; 196	

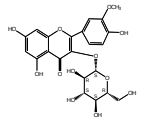
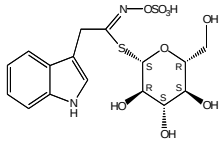
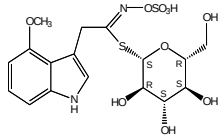
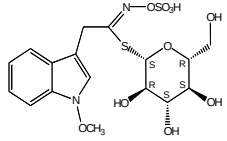
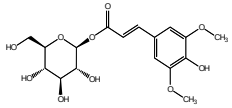
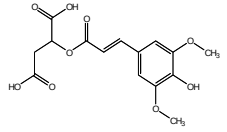
6 APPENDIX B

Structure identification of the most relevant flavonoid glycosides found in Arabidopsis root extract

Peak old No	Peak No	Rt [min]	MW	(–)-ESI	(–)-MS/MS	UV-ABS [nm]	Structure	CAS-nr
Triglycosides	**Triglycosides**							
7	**1**	15.4	740	739	593, 430, 429, 284	347, 328, 283min, 267, 248min	**K-neohesperidoside-3-R-7** [Kerhoas et al.; Col & tt7]		not found
	–	20.5	740	739	**593**, 285	347; 332S; 285min; 267; 248S; 238min; 222S; 198	**K-R-G-3-R-7 or K-rutinoside-3-R-7** (ref14)		57526-56-4
3	**2**	13.6	756	755	**609**, 489, 463, 447, 446, 343, 325, 301, 300, 299, 271	353, 288min, 264, 255	**Q-neohesperidoside-3-R-7** [Stobiecki et al.; Col; WS; d1; d2d2]		not found
	–	14.1	756	755	**593**, 327, 285	347, 324S, 285min, 267, 238min, 220S, 198	**K-R-G-3-G-7 or K-rutinoside-3-G-7** (ref16)		34336-18-0
	–	14.3	756	755	**609**, 429, 285, 284	345; 324S; 287min; 365; 245S; 238min; 220S; 197	**K-sophoroside-3-R-7** (ref10)		93098-79-4
10a	**3**	17.8	756	755	**609**, 447, 301, 271	354, 283min, 265, 256	**Q-R-G-3-R-7 or Q-rutinoside-3-R-7** [Veit used wrong K–3-Ruti-7-R label; litt Stobiecki wrong; ColR, d2d2R, WS R]		57528-70-8
10b	–	18.3	756	755	**609**, 285	348, 282min, 267, 248min, 225S, 198	**K-G-G-3-R-7 or K-gentiobioside-3-R-7** [Veit et al.; **tt7**; WS R; d1R]		not found
–	**–**	8.6	772	771	**609**, 429, (327), 285, 284, (257, 255)	346; 327S; 286min; 266; 247S; 238min; 222S; 197	**K-sophoroside-3-G-7** (ref13)		55136-76-0

7	**4**	15.8	772	771	625, 609, 463, 447, 301	348, 302min, 293, 263, 251S, 238min, 215, 203	**Q-G-G-3-R-7** [Stobiecki et al.; ColR; d1d2R; d2R] (gentiobioside or sophoroside)		64828-40-6 (sophoroside) - gentiobioside unknown
Diglycosides	**Diglycosides**								
18	**5**	23.9	578	577	431, **285**	343, 320, 282min, 263, 245min	**K-R-3-R-7** [Kerhoas et al.; tt7; WS]		not found
9	**6**	16.8	594	593	447, 431, 327, **285**, 255	340, 320, 285min, 264, 250min	**K-R-3-G-7** [Kerhoas et al.; tt7 & rol1-1]		not found
13	**7**	20.6	594	593	**447**, 431, 285, 256	346, 325, 285min, 265, 245min	**K-G-3-R-7** [Kerhoas et al.; WS & tt7] cf ref15 & Veit		2392-95-2
15	**9**	20.8	594	593	**447**, 431, 301	(348, 285min, 263, 256, 238min)	**Q-R-3-R-7** [Kerhoas et al.; rol1-1; (WS)]		not found
–	**–**	25.8	594	593	**285**	320S; 288; 247min; 224S; 211; 205min; 195	**K-Rutinoside-3** or K-R-G-3 [ref2 & Veit]		17650-84-9
5	**8**	14.2	610	609	489, **447**, 327, 285, 247	(332, 273min, 240, 228, 214min)	**K-G-3-G-7** (rol1-1 & tt7; ref17)		25615-14-9
11	**10**	18.1	610	609	463, **447**, 343, **301**, 299, 271	353, 284min, 265, 257, 236min	**Q-G-3-R-7** [Kerhoas et al.; Stobiecki et al.; Col & WS (lblc8471); Q-R-G-3 excluded]		18016-58-5

17???	–	22.5	610	609	343, **301**, 300, 271, 255, 179	350, 278min, 263, 255 (weak)	**Q-R-G-3** (ref1&22 rutin)		153-18-4
16	**11**	21.3	624	623	**477**, 461, 315, 301, 300	355, 286min, 265, 255, 237min	**I-G-3-R-7** [Stobiecki et al.]		17331-71-4
–	–	26.2	624	623	**315**, 300, 271, 255, 243	354; 311S; 277min; 264S; 254; 237min; 206	**I-Rutinoside-3** or I-R-G-3 or Narcissin [ref3]		604-80-8
Monoglycoside: Monoglycosides									
19	**12**	31.2	432	431	327, **285**, 255	325, 277min, 265, 253min, 220 (week absorption!!)	**K-R-3** [Kerhoas et al.; tt7 (Iblc9843)]		482-39-3
–	–	32.9	432	431	**285**, 284, 257, 151	364, 331, 287min, 267, 255, 237min, 218, 198	**K-R-7** (ref-19)		20196-89-8
19	**13**	27.3	448	447	327, 301, **285**, 284, 255, 227, 151	345, 290, 280min, 265, 247min	**K-G-3** [Kerhoas et al.; tt7] & (ref-Veit)		27614-89-7 (not fully defined)
–	–	27.6	448	447	**301**; 271; 255; 179; 151	337; 300S; 282min; 268; 247min; 212; 203min; 196	**Q-R-3** [Kerhoas et al., WS; pgp1x19 (Iblc8474) contains both Q-R-3 & K-G-3; Extrasynth; ref9]		522-12-3
20	**14**	23.9	464	463	**301**	353, 280min, 263, 253, 243	**Q-G-3** [Routaboul et al.]		482-35-9

20	**15**	27.9	478	477	387, 357, 315, **314**, 300, 299, 286, 285, 271, 257, 243, 179, 151	353, 280min, 263, 253, 243	**I-G-3** (ref-ExtraSynth)		5041-82-7
Undefined	**Non flavonoids**								
1	**17**	11.5	448	447	367, 275, 269, **259**, 241, 205, 195, 139	288, 282, 273, 250min, 220	**Glucobrassicin** [Le Gall et al.]		4356-52-9
8	**18**	15.3	478	477	325, 299, 275, 259, 241, 235, 199, 171	342, 305min, 283, 266, 254????	**4-Methoxyglucobrassicin (4-Methoxy-3-indoylmethyl)** [Le Gall et al.]		83327-21-3
14	**19**	20.3	478	477	447, **446**, 429, 285, 284, 259, 241, 224, 195, 163	347, 324, 283min, 266, 248????	**Neoglucobrassicin (N-Methoxy-3-indoylmethyl)** [Le Gall et al.]		5187-84-8
–	**–**	14.3	486	485	223, 205, 247, 265, 190, 325, 295, 164	332, 230/240	**Sinapoyl glucose** [Stobiecki et al.]		78185-48-5
–	**–**	24.4	340	223	223, 208, 164	330, 230/240	**Sinapoyl malate** [Stobiecki et al.]		92344-58-6
–	**16**	30.2	490	489			**Unknown**		

Literature: Kerhoas et al. *J. Agric. Food Chem* , **2006**, *54,* 6603-12
Routaboul et al. *Planta* , **2006**, *224,* 96-107
Stobiecki et al. *Metabolomics,* **2006**, *2* , 197-219
Le Gall et al. *Metabolomics,* **2005**, *1* , 181-198
Veit et al. *J. Nat. Prod.* **1999**, *62,* 1301-1303

7 CURRICULUM VITAE - JANUARY 2008 -

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Education and Research experience

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29th November 2006: PhD thesis defense at the University of Zürich, Institute of Plant Biology, Zürich, Switzerland.

Sept 2002 - Jan 2007: PhD student at the University of Zürich, Institute of Plant Biology, Laboratory of Plant Molecular Physiology. Supervisor: Prof. Enrico Martinoia. Research topic: *"Root flavonoids: their transport and role in intra and extra cellular signaling"*

May 2001 - Aug 2002: Post-diploma research fellowship from the University of Milan "for the training of the young most promising students in scientific and technological research", laboratory of Plant Molecular Genetics, supervisor: Prof.Enrico Mario Pè. Title of the project: *"Organization and evolution of the Glutathione S-transferase multigene family in Arabidopsis thaliana"*

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Feb 1999 - Apr 2001: Experimental diploma thesis in the laboratory of Plant Molecular Genetics (University of Milan). Thesis title: "*Genetic and physiological approaches to study cadmium stress responses in B. Juncea"*. Supervisors: Prof. Mario Enrico Pè and Prof. Gian Attilio Sacchi

Nov 1994 - Apr 2001 Student in *Agricultural Sciences* at *Università degli Studi di Milano* (I)

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Sept 2001- July 2002: Part-time surveying teacher at secondary school "Istituto Polifunzionale A. Sant.Elia" (Istituto Tecnico per Geometri e Ist. Professionale perl'Industria e l'Artigianato), Cantù (CO), Italy. Member of III examining board of State Examination for Surveyor.

Sept 2000 - July 2001: Part-time surveying teacher at secondary school "Istituto Tecnico Statale per Geometri, L. Nervi" Varese, Italy. Member of IV examing board of State Examination for Surveyor.

1995 - 1998: Botanical guide in the orchard and in the botanical garden of "Fondazione per il Miglioramento dell'ortofloro-frutticoltura di Minoprio" Como, Italy.

Scientific research and Technical expertise

- **Basic methods in molecular biology and biochemistry:** nucleic acid and protein extraction from plant tissues and bacteria/yeast cells**,** PCR, DNA electrophoresis, restriction enzyme digests, cloning of DNA fragments, RT-PCR, *southern, northern, western blots* analyses, cDNA library construction and screening, heterologous protein expression and purification in *E. coli.*

- **Yeast molecular genetics and biochemistry:** yeast transformation, expression of recombinant proteins, drop tests, colony survival assay, transport assay.

- **Plant biology, molecular biology and biochemistry:** hydroponic cultures for rice, maize, *B. juncea, Lupinus albus* and *Arabidopsis thaliana*, Arabidopsis cell cultures, Arabidopsis and barley protoplast-vacuole preparation and transport assays, green tissue mineralization for ash analysis, onion epidermis and protoplasts bombardment for transgene subcellular localization, *Agrobacterium*-mediated stable transformation, confocal microscope analysis of biological specimens, *in vivo* flavonoid localization, thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC) analysis of secondary metabolites of *Lupinus albus* and *Arabidopsis thaliana*, HPLC-ESI-MS/MS analysis and structural elucidation of *Lupinus albus* and *Arabidopsis thaliana* flavonoids, starch extraction and measurement from *Arabidopsis thaliana* leaves.

- **Bioinformatics:** Routine usage of programs of *Microsoft Office package*. Good knowledge of bioinformatics tools for nucleotide and amino acidic sequence analysis (Clustal, BioEdit, Phylip, Gene scan, Promoter scan, etc.). Good expertise in using on-line databases.

- **Languages:** home language Italian; good level of English speaking and writing; basic level of German speaking and writing.

Courses attendance

Courses attended at the Plant Science Center Zurich-Basel during doctoral studies:

- Functional Genomics: Introduction to transcriptional profiling and proteomic
  Dr. Hennig L. and Dr. Baginsky S. (11th.-12th. May 2004)
- Spectrum in plant sciences
  Winter semester 2004/2005
- Epigenetic Regulation in Plants
  Prof. Grossniklaus U. Prof. Gruissem W, Dr. Hennig L., Dr. Köhler C., Prof. Meins Jr., Prof. Richmond T. (29th-30th Juanuary 2004)
- Transport processes in plants
  Prof. Martinoia E., Dr. Geisler M., Dr. Klein M., Dr. Sujeoung S. (12th-13th February 2004)
- Web Publishing
  Dr. Paschke M. (3rd-4th November 2004)
- Introduction to microscopy
  Roland Gebert, Martin Müller, Christof Sautter, Lloyd Vaughan (7th-11th February 2005)

Courses attended at the NCCR Plant Survival Graduate School of Neuchâtel during doctoral studies:

- How to Make Scientific Presentations and Poster Interesting?
  Prof. McNeil J. (12th-14th March 2003)
- Effective Public Speaking
  Prof. Goldschimd M.L. (19th April and 21st June 2004)
- Planning a career strategy – Part1 – Job finding methodology and networking
  Dr. Pavel Kraus (8th and 9th November 2005)
- Preparing for the next step – Improving your CV and practical training to meet your future employer's expectations
  Dr. Pavel Kraus (16th and 17th January 2006)
- Scientific Bibliography and Communicating Science
  Prof. Dreier F., Dr. Smrekar O., Dr. Pohl C. (7th February 2003)
- Identifiying Natural Compounds by Gas and Liquid Chromatography Couled to Mass Spectrometry
  Prof. Krock B, Prof. Tabacchi R., Dr. Abou-Mansour A. (18th June 2003)
- Microarrays: Techniques and Data Mining (Affymetrix GeneChip Analysis)
  Prof. Goldstein D, Dr. Delorenzi, Dr. Reymond P., Prof. Hagenbuchle O., Zimmermann P., Renou JP (27th-28th January 2005; 14th January and 4th February 2005)
- Plant Metabolism, Growth and Development
  Prof. Smith SM. And Dr. Zeeman S.C. (12th-14th February 2003)
- Genetics of Biodiversity and Applications
  Prof Cronk Q., Prof. Koornneef M., Prof Zamir D. (15th-17th March 2004)

Courses modules attended within the program of "Fix the leaky pipeline" tailored to women scientist with PhD and organized by the ETH Domain's Group for Equal Opportunities:

- Module no 5: Leadership skills and people management in the academic world, 6th-7th March 2008, EMPA, Dübendorf. Cost: 650 chf., financially supported by the NCCR Plant Survival of Neuchâtel

- Module no 6: How to succeed in the scientific community, 8th-9th April 2008, ETHZ, Zürich. Cost: 650 chf., financially supported by the NCCR Plant Survival of Neuchâtel

Meetings and Workshops attendance

- 13th International workshop on plant membrane biology, Montpellier, France, July 6th-10th, 2004. Poster presentation: "What is the role of MATE (Multidrug And Toxic compound Extrusion) transporters in white lupin plants growing in sparingly available phosphorus?" **Santelia D.**, Weisskopf L., Tomasi N., Abou-Mansour E., Tabacchi R., Martinoia E.

- NCCR Plant Survival International Conference, Leysin, Switzerland, March 31st-April 3rd 2005. Poster presentation: "Characterization of an ABC transporter affecting root hairs elongation" **Santelia D.**, Geisler M., Vincenzetti V., Bovet L., Martinoia E.

- Tri-National Arabidopsis Meeting, – joint workshop NCCR & 3éme cycle romand en science biologiques, Neuchatel, CH 24th- 27th August 2005. Poster presentation: "The Arabidopsis ABC transporter AtPGP4 is involved in lateral root and root hair development" **Santelia D.**, Vincenzetti V., Bovet L., Geisler M., Martinoia E.

- PhD Symposium "Feeding the needs of tomorrow- Plant Science from basic application", University of Zürich, Switzerland, 31st March 2006. Poster presentation: "MDR-like ABC transporter AtPGP4 is involved in lateral root and root hair development" **Santelia D.**, Geisler M., Vincenzetti V., Mancuso S., Bovet L., Martinoia E.

Publication list

Santelia D., Vincenzetti V., Azzarello E., Bovet L., Fukao Y., Duchtig P., Mancuso S., Martinoia E., Geisler M. (**2005**) "MDR-like ABC transporter AtPGP4 is involved in auxin-mediated lateral root and root hair development" *FEBS Lett.* **24**, 5399-5406

Weisskopf L., Abou-Mansour E., Fromin N, Tomasi N., **Santelia D.**, Edelkott I., Neumann G., Aragno M., Tabacchi R., Martinoia E. (**2006**) "White lupin has a

complex strategy to limit microbial degradation of secreted citrate required for phosphate acquisition" *Plant, Cell and Environment* **29**, 919-927

Weisskopf L., Tomasi N., **Santelia D.**, Martinoia E., Langlade N., Tabacchi R., Abou-Mansour E. (**2006**) "Isoflavonoid secretion from white lupin roots is influenced by phosphate supply, root type and cluster root stage" *New Phytologist* **171**, 657-668

Badri D.V., Loyola-Vargas V.M., Broeckling C.D., De-la-Pena C., Jasinski M., **Santelia D.**, Martinoia E., Lloyd W.S., Banta L.M., Stermitz F., Vivanco J.M. (**2008**)"Altered profile of secondary metabolites in the root exudates of Arabidopsis ATP-Binding Cassette (ABC) transporter mutants" *Plant Physiology* **146**, 762-771

Ringli C., Bigler L., Leiber R.M., Diet A., **Santelia D.**, Frey B., Pollmann S., Klein M. "The modified flavonol glycosylation profile in the Arabidopsis *rol1* mutants results in auxin-induced and auxin-independent alterations in plant growth and cell shape formation" *in revision by The Plant Cell, resubmission encouraged*

Santelia D., Vincenzetti V., Henrichs S., Sauer M., Bigler L., Klein M., Yangsook L., Friml J., Geisler M., Martinoia E. "Flavonoids redirect PIN-mediated polar auxin fluxes during root gravitropic responses" *in revision by The Journal of Biological Chemistry, minor revisions asked*

Bailly A., Sovero V., Vincenzetti V., **Santelia D.**, Bartnik D., Koenig D.W., Mancuso S., Martinoia A., Geisler M. "Drug-mediated modulation of P-glycoprotein-mediated auxin transport conferred by interaction with immunophilins" *in revision by The Journal of Biological Chemistry, minor revisions asked*

Santelia D., Bigler L., Vincenzetti V., Geisler M., Ringli C., Keller F., Klein M., Martinoia E. "Flavonoid accumulation in the root elongation zone is modulated by auxin", *in preparation*

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